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HPLC metody stanovení benzokainu a jeho rozkladných produktů v Herplex gelu

DIPLOMOVÁ PRÁCE

ve spolupráci s
UNIVERZA V LJUBLJANI
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HPLC methods for measuring benzocaine and its degradation products in Herplex gel

DIPLOMA THESIS

in cooperation with
UNIVERSITY OF LJUBLJANA
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CONTENT

ABSTRACT

Benzocaine as a main active substance in the Herplex gel, Galex d.d. used for healing of fever blister is treated as a local anaesthetic. According to European Pharmacopoeia titration method for determination of benzocaine is applied in the pharmaceutical industry. However its degradation products (determination of *p*-nitrobenzoic acid ethylester (EPNB) and *p*-aminobenzoic acid (PABA)) are not described in pharmacopoeias (neither Ph. Eur nor USP).

Two high performance liquid chromatography methods with UV detection were developed for determination of benzocaine and both degradation products (*p*-nitrobenzoic acid ethylester and *p*-aminobenzoic acid). Procedures for extraction of these active from the Herplex gel were developed too.

Optimum results were obtained with a column Eclipse XDB – C 18 (4.6 x 150 mm, 5 μm) and a mobile phase comprising glacial acetic acid solution (10%, v/v) and methanol. Injection volume was set to 10 μml and temperature to 40 ˚C. Other conditions used in methods varied. The first method for benzocaine and EPNB was set to flow rate 2 ml/min, detection time 30 min and mobile phase was carried as a gradient profile programme whereas the second method for PABA was set to flow rate 1 ml/min, detection time 10 min and glacial acetic acid solution and methanol in the rate of 90 : 10%, v/v. Both methods were validated (specificity, linearity, accuracy and precision) at single wavelength 258 nm. Limit of detection and limit of quantification values for degradation products EPNB and PABA were determined. For EPNB LOD value 0.3404 μg/ml and LOQ value 1.0315 μg/ml were found and for PABA LOD value 0.1691 μg/ml and LOQ value 0.5123 μg/ml were found.

ABBREVIATIONS

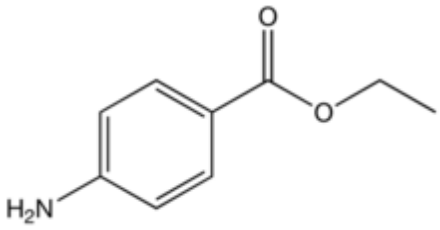
BZC	benzocaine
c	mass concentration (μg/ml)
CV	coefficient of variation
DCHM	dichloromethane
DNA	deoxyribonucleic acid
EPNB	*p*-nitrobenzoic acid ethylester
FDA	US Food and Drug Administration
HPLC	high performance liquid chromatography
ICH	International Conference on Harmonization
LOD	limit of detection (μg/ml)
LOQ	limit of quantification (μg/ml)
M	molar concentration (mol/l)
MeOH	methanol
PABA	*p*-aminobenzoic acid
Ph. Boh.	Pharmacopoeia Bohemica
Ph. Eur.	European Pharmacopoeia
R^2	correlation coefficient
R.S.D.	relative standard deviation
S	slope of the calibration curve
S.D.	standard deviation
SST	system suitability test
THF	tetrahydrofuran
USP	United States Pharmacopoeia
UV	ultraviolet
VF	volumetric flask

1 INTRODUCTION

1.1 BENZOCAINE

Benzocaine (Benzocainum)

Benzocaine, 4-aminobenzoic acid ethylester or *p*-aminobenzoic acid ethylester ($C_9H_{11}NO_2$) is a white, crystalline powder or in form of colourless crystals, very slightly soluble in water, freely soluble in alcohol 96% and in ether. Its molar mass is 165.189 g/mol [1, 2, 3]. Its monograph can be found in Ph. Eur 5th Ed., USP XXII and Ph. Boh. 2002.



Benzocaine belongs to drug category of anaesthetics, antipruritics and local anaesthetics (ester type). Benzocaine is included in many pharmaceutical topical preparations (rarely cosmetics) [4] commonly used in the treatment of wound, pains, itches caused by conditions such as sunburn or other minor burns, insect bites or stings, minor cuts and scratches but also in the treatment of larynx, buccal affections, nasal cavity, respiratory tract or trachea, otitis externa, dermatitis, haemorrhoids pruritus, urinary tract or vagina and rectum [5].

Mechanism of benzocaine action is based on the binding to sodium channel and reversibly stabilization of the neuronal membrane which decreases its permeability to sodium ions since pain is caused by the stimulation of nerve endings [5]. When the nerve endings are stimulated, sodium enters the nerve ending, which causes an electrical signal to build up in the nerve. Once the electrical signal becomes big enough, it is able to travel to the brain, which then interprets this as pain. Therefore

blocking the initiation and conduction of nerve impulses inhibits depolarization of the neuronal membrane [6].

Topical anaesthetics with benzocaine are available in creams, ointments, aerosols, sprays, lotions and gels. Preparations containing benzocaine and another local aesthetic are for example [4]:

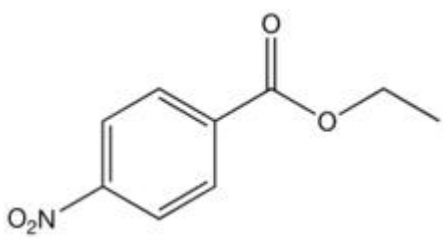
- Wound and burn preparations
- Sunburn remedies
- Haemorrhoidal preparations
- Oral and gingival products
- Sore throat sprays
- Callous and wart remedies
- Creams for treatment of poison ivy
- Toothache and denture irritation products

A considerable number of publications have appeared describing different strategies to quantify benzocaine in tissues and biological fluids of several fish species. Also there are papers for the determination of the active in different pharmaceutical dosage forms such as tablets, solutions and suspensions, semisolid preparations, suppositories, suncreems and after-sun products [7].

1.2 BENZOCAINE DEGRADATION PRODUCTS

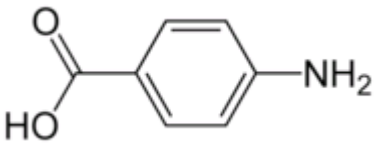
***p*-Nitrobenzoic acid ethylester**

p-Nitrobenzoic acid ethylester or 4-nitrobenzoic acid ethylester (EPNB) is a white crystal, slightly soluble in water with formula $C_9H_9NO_4$ and molar mass 195.175 g/mol. *p*-Nitrobenzoic acid ethylester is not listed in pharmacopoeias [8].



p-Aminobenzoic acid

p-Aminobenzoic acid, 4-aminobenzoic acid, PABA or vitamin B_x is a white crystalline substance, slightly soluble in water with formula $C_7H_7NO_2$ and molar mass 137.136 g/mol. PABA is not listed in pharmacopoeias [9].



PABA is a non-protein amino acid that is widely distributed in nature. It is sometimes referred to vitamin B complex (vitamin B_x), but it is neither a vitamin nor an essential nutrient for humans. PABA is an intermediate in the synthesis of folic acid in bacteria. The sulphonamide antibiotics are structurally similar to PABA and interfere with the synthesis of nucleic acids in sensitive microorganisms by blocking the conversion of PABA to the co-enzyme dihydrofolic acid, a reduced form of folic acid. In humans, dihydrofolic acid is obtained from dietary folic acid; thus sulphonamides do not affect human cells [10]. Food sources of PABA are animal meat (liver, kidney, etc.), brewer's yeast, molasses, whole grains, mushrooms and spinach, and can be made by intestinal bacteria.

PABA is used to improve the protein used in the body, it relates to red blood cell formation as well as assisting the manufacture of folic acid in the intestines. Furthermore PABA is included in sunscreen preparations since it helps to protect the skin from sunburn and cancer (antioxidant) [12]. However, it has been determined that it increases the formation of a particular DNA defect in human cells, thus increasing the risk of skin cancer in people who lack the mechanisms to repair these cellular defects. PABA supplementation may help return gray hair to its original colour if the change was caused by either stress or a nutritional deficiency. [11] Pharmaceutical doses of PABA are indicated for Peyronie's disease, scleroderma, morphea and linear scleroderma [10].

PABA is passively absorbed mainly from the small intestine. From there, it enters the portal circulation. Some metabolism of PABA occurs in the liver. A major metabolite is N-acetyl PABA. PABA and its metabolites are mainly excreted in the urine. Small amounts are eliminated in the feces and in bile, milk and other secretions [10].

When PABA is in short supply fatigue, irritability, nervousness and depression might manifest itself as well as constipation. Weeping eczema has also been noted in people with PABA deficiency as well as patchy areas on the skin [12].

Symptoms of high intake were found when higher than factor 8 sunscreens are used; the manufacture of vitamin D in the body might be reduced. Nausea, skin rashes and vomiting might be indicative of PABA taken in excess. Excessive levels of PABA are stored in the body and may cause liver damage [12].

1.3 HERPLEX GEL

Herplex gel produced by Galex d.d., Murska Sobota falls into group of non-prescription medicines. These medicines are safe and effective for application without a doctor's prescription and perform considerable part in therapy of ailments as cold and flu, nausea or travel sickness, constipation as well as relieve pain, cure of sports injuries, musculoskeletal problems, allergies and hay fever and many others.

Herplex is used for treatment of fever blister caused by *Herpes simplex* virus. Ointment provides a soothing effect, as it reduces the sensation of pain and tension of the skin. Zinc oxide contained dries up, protects the damaged skin and facilitates its regeneration [13].

1.4 ANALYSIS OF BENZOCAINE AND ITS DEGRADATION PRODUCTS

The method for quantification of benzocaine and its degradation products (EPNB and PABA) in a bioadhesive gel by HPLC developed by Pérez-Lozano et al. [7] was applied for extraction from the Herplex gel.

The validated study was performed with a high-performance liquid chromatographic system consisted of a column oven, a quaternary pump, an automatic injector and a DAD detector. The DAD detector was set at 258 nm. Data acquisition was made out using a chromatography software package.

Chromatographic separation was performed using a Nucleosil 120 C18 column 250 x 4.6 mm, 10 µm particle size. The mobile phase consisted of methanol and solution of glacial acetic acid (10%, v/v) that were carried as a gradient programme (Table 1). Both methanol and glacial acetic acid solution were degassed by filtering through a 0.45 µm GH-membrane filter. The flow rate was 2.0 ml/min. The DAD detector was operated at 258 nm. The injection volume was 10 µl. During the analysis the column was equilibrated at 40 ˚C. Each determination required 30 min.

Table 1

Gradient profile programme to carry out the chromatographic method [7]

Time (min)	Buffer solution (%)	Methanol HPLC (%)
0	90	10
9	90	10
13	60	40
25	60	40
28	90	10
30	90	10

Validation parameters as specificity, linearity, intra-day and intermediate precision, accuracy and robustness of method were determined. It was checked that benzocaine raw material as well as benzocaine reference standard elute at the same retention time indicating a positive identification of the drug. All the compounds of the injected

sample elute at different retention times and do not interfere between them. Linear relationship, with a correlation coefficient higher than 0.9990, was obtained between the peak areas of benzocaine at the corresponding concentrations in a range of 70-130% of the benzocaine. The factors selected to examine of robustness were the wavelength (nm), temperature (˚C), flow rate (ml/min), mobile phase (percentage methanol) and volume of injection (µl). It was proved that the selected factors remained unaffected by small variations of these parameters. The LOD (LOD for PABA was 5.03 µg/ml and for EPNB was 6.06 µg/ml and the LOQ values (LOQ for PABA was 5.03 µg/ml and for EPNB was 7.99 µg/ml) for both degradation products EPNB and PABA were obtained.

1.5 VALIDATION OF HPLC METHOD

Method validation, as an important part of method development, is the process ofdemonstrating that analytical procedure is suitable for its intended purpose. Validation of a method tests its ability to achieve accuracy, specifity, reliability and precision over the specified range that an analyte will be analyzed.

Several guidelines were edited to provide framework and definitions of validation issues and requirements for control of the quality of proposed method, e.g.:

- The US Food and Drug Administration (FDA) [14]:
  Guideline for Submitting Samples and Analytical Data
  for Methods Validation,
- International Conference on Harmonization (ICH) [15]:
  Validation of Analytical Procedures: Text and Methodology,
- The US Pharmacopeia (USP) [16].

The most important analytical procedures that require validation are [15, 16]:

- Identification tests (Category IV in USP) – analytical methods for guaranteed the identity of an analyte in a sample normally achieved by comparison of a property of the sample (e.g. spectrum, chemical reactivity, etc) to that of a reference standard.

- Quantitative and limit tests of impurities (Category II in USP) – analytical method for determination of impurities in a sample or degradation compounds in finished pharmaceutical products.
- Assay of active or other ingredients (Category I in USP) – analytical methods for quantitative measurement of the major components in the drug substance or drug product.
- (Category III in USP) – analytical methods for determination of performance characteristics (e.g. dissolution, drug release)

ICH parameters for validation of analytical procedures are:

- Specificity (Selectivity in USP, FDA)
- Linearity
- Range
- Accuracy
- Precision (Repeatability, Intermediate Precision, Reproducibility)
- Detection Limit
- Quantification Limit
- Robustness

FDA Reviewer Guidance [17] uses additional analytical performance characteristics, e.g. recovery, sensitivity and sample solution stability. FDA guideline defines the system suitability tests as a required parameter for assay validation.

ICH guidelines treat the system suitability tests as a part of validation parameters whereas the USP describes system suitability tests in a separate chapter <621>. Ruggedness is comprised in USP list of validation parameters.

Table 2

ICH and USP validation characteristics [18]

Procedure / Parameter	Assay	Testing for impurities		Identification	
		Quantitative	Limit tests	ICH	USP
Specificity (2)	Yes	Yes	Yes	Yes	*
Linearity	Yes	Yes	No	No	*
Range	Yes	Yes	*	No	*
Accuracy	Yes	Yes	*	No	*
Precision	Yes (1)	Yes (1)	No	No	Yes
LOD	No	No (3)	Yes	No	*
LOQ	No	Yes	No	No	*

* May be required, depending on the nature of the specific test (FDA)

(1) In cases where reproducibility has been performed, intermediate precision is not needed

(2) Lack of specifity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(3) May be needed in some cases

SPECIFICITY

Specificity (Selectivity) is the ability of an analytical method to differentiate the analyte of interest in the presence of all potential sample components [14]. The discrimination is evaluated by the comparison of the response of a blank sample without the analyte (only matrix, excipient) with the response of a sample containing the analyte and with the response of a sample spiked with the analyte, degradation products, process impurities, related chemical compounds, synthesis intermediates, etc.

In order to assure the acceptability of specifity studies the analyte is observed under various stress conditions such as 0.1 M HCl; 0,1 M NaOH; heat, UV light, oxidant (H_2O_2) and the results are compared.

LINEARITY

Linearity verifies that the sample solutions are directly proportional to concentration in a concentration range where analyte response is linear [19]. Standard solutions can be prepared by a serial dilution of mother solution or by an independent weighing. According to ICH guidelines the calibration curve is generated by a minimum of 5 concentration levels of the target analyte concentration while FDA recommends 6-8 non-zero standard solutions (in triplicates). A calibration curve for each analyte in the sample is required.

The linearity of analytical method is determined by the linear regression equation (1) with the y-intercept (less than a few percent of the response obtained for the analyte at the target level) and the slope (S) of linear regression line, the correlation coefficient ($R^2 > 0.999$).

$$y = ax + b \qquad (1)$$

a slope of the calibration curve (S)

b y-intercept

RANGE

Range is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written [16].

The range of the analytical method usually depends on an intended purpose of the method. Typical ranges for the assay are 80-120% of the target concentration, for impurity test the minimum range is from the reporting level of each impurity to 120% of the specification [19], +/- 20% over the specified range for dissolution testing and 70-130% of the expected concentration for content uniformity testing [15]. The range is normally expressed in the same units as the results obtained in the linearity study.

ACCURACY

Accuracy is the degree of closeness between the true value of analytes in the sample and the experimental value determined by the new method [15]. The most common approach for an evaluation of accuracy is based on the recovery of known amounts of analyte spiked into blank sample. Other approaches use, for example, second existing validation method with assessed accuracy or reference material with known value. In cases where is impossible to generate a pure sample without the analyte the accuracy is performed by addition of known quantities of standard (lyophilized material, etc.).

According to ICH the accuracy should be determined by at least in triplicate at each level (80, 100 and 120% of label claim), according to FDA a minimum of 5 determinations for at least 3 concentrations (low, medium and high) in the range of expected concentrations. For impurity methods, spiked samples are prepared in triplicate at 3 levels over a range that covers the expected impurity content of the sample [19].

The accuracy should be proved throughout the same procedure as will be used in the final method procedure. It is assessed as a percent recovery (R_i) in relation to the known amount of analyte added to the sample (2).

$$R_i(\%) = 100 \cdot \frac{c_i}{c_o} \qquad (2)$$

c_i found or measured concentration

c_0 added concentration

PRECISION

The precision is the degree of agreement among a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [15]. The precision is calculated as standard deviation (S.D.), relative standard deviation (R.S.D.) named also coefficient of variation (CV) by using formulas listed below (3, 4 and 5). Both precision and accuracy can be calculated from the same analytical experiments. The acceptance criteria for precision depend much on the type of analysis.

Precision may be divided to these categories [15]:

- REPEATIBILITY (Intra-day precision)

  Repeatability is the measurements of the sample under the same operating conditions over a short interval of time (the same laboratory, analyst, equipment). According to ICH samples of 3 different concentrations (low, medium and high) at three determinations are used for evaluation or samples of 100% concentration at 6 determinations. According to FDA analysis of a minimum of 5 determinations at three different concentrations (low, medium and high) is recommended and the precision values at each concentration should not exceed 15% of the CV for bio analytical procedures. But, in general, the claim for the precision is 1-5% of the CV.

- INTERMEDIATE PRECISION

  Intermediate precision evaluates within lab variations and their effects to the submitted method. Variations involve using different equipment, analysts, day, sources of reagents, columns, but in one laboratory.

- REPRODUCIBILITY

  Reproducibility is determined by testing homogeneous samples in multiple laboratories (collaborative studies). Reproducibility is not normally part of submitted method if the intermediate precision is carried out.

$$S.D. = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \quad (3) \qquad R.S.D.(\%) = \frac{100}{\bar{x}} \cdot \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \quad (4)$$

x_i individual measurement in a set of measurement

$\bar{x}$ arithmetic mean of the set

n number of individual measurements

$$CV = \frac{S.D.}{\bar{x}} \quad (5)$$

DETECTION LIMIT (LOD)

The limit of detection is the lowest concentration of analyte in a sample that can be detected but not necessarily quatitated as an exact value [15]. It is usually expressed as the concentration of analyte in the sample.

The type of procedure influences the determination of LOD. Non-instrumental methods detect LOD by visual evaluation (e.g. thin-layer chromatography). These methods analyse the samples with known concentration of analyte and establish the minimum level at which the analyte can be reliable detected. Instrumental procedures use a detection based on signal-to-noise ratio or on the standard deviation (S.D.) of the response and the slope (S) of the calibration curve according to the formula (6). The S.D. of the response can be determined based on the S.D. of the blank, on the R.S.D. of the regression line, or the S.D. of y-intercepts of regression lines. A signal-to-noise ratio is comparing of signal of samples with known low amount of analyte with signals from the analytical blank samples [15]. Ratio 3:1 or 2:1 is generally accepted. It is important to establish the LOD on all instruments, in each laboratory to which the method will be transferred.

$$LOD = \frac{3.3 \times S.D.}{S} \qquad (6)$$

S.D. standard deviation of y-intercepts of regression lines

S slope of the calibration curve

QUANTIFICATION LIMIT (LOQ)

The limit of quantification is the lowest level of analyte in a sample that can be quantitatively determined with suitable precision and accuracy [15]. The LOQ is expressed as the concentration of analyte in the sample.

Determination of LOQ also depends on the procedure. Non-instrumental approach uses visual evaluation for the analysis of samples with known concentrations of analyte and for establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. Also signal-to-noise ratio is performed for instrumental procedures where ratio should be 10:1. The LOQ may be calculated from the standard deviation of the response and the slope. It takes the form (7):

$$LOQ = \frac{10S.D.}{S} \qquad (7)$$

S.D. standard deviation of y-intercepts of regression lines

S slope of the calibration curve

ROBUSTNESS

Robustness of the method investigates its ability to remain unaffected in variations in method parameters. Parameters that could be validated are pH of the mobile phase, buffer concentration of the mobile phase, percent organic content, flow rate, different columns and temperature.

RUGGEDNESS

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different temperature, different days, etc [16].

RECOVERY

The recovery is amounts of analyte obtained from a blank sample spiked with known quantity of standards in matrix compared to the true aqueous sample spiked with standards of the same quantity that represent 100% recovery. The recovery should be detected by a minimum of 6 determinations for at least 3 concentrations (low, medium and high) in a range of expected concentrations [14].

SAMPLE SOLUTION STABILITY

The stability tests of an analyte or stress studies should be provided to demonstrate that impurities and degradants from the active ingredient and excipients, the storage conditions and the container systems do not interfere with the active ingredient. The solutions should be stable enough to allow for delays such as overnight analyses using auto samplers. Stability procedures should reflect conditions during actual sample handling and analysis [14]. The limits of stability are gained by comparison to freshly prepared standards.

Examples of stability tests define in FDA guidelines (at least 3 measurements of each of the low and high concentrations):

- Short-Term Temperature Stability – analysis of stability after thaw at room temperature and storing 4-24 hours
- Long-Term Stability – evaluation of stability after storing at least the time between the date of first sample collection and the last sample analysis.
- Stock Solution Stability – determination of stability of stock solutions after storing at room temperature for at least 6 hours.
- Post-Preparative Stability – assessment of stability in the final extract during the expected maximum analysis time.
- Freeze and Thaw Stability – valuation of stability after three freeze and thaw cycles.

Acceptable stability is 2% change in standard or sample response, relative to freshly prepared standards for assay methods or 20% change for impurity methods [19].

1.6 SYSTEM SUITABILITY TEST

System suitability tests (SST) [14, 18, 20] are used to ensure that the complete testing system (including instrument, reagents, columns, analysts, etc.) is suitable for the intended application and is working properly at the time of analysis. According to the ICH and the FDA guidelines, SST is an integral part of analytical procedures and should be establish at the beginning part of method validation to assure that the HPLC system and procedure are capable of carrying out the analysis from the original laboratory to another, on other equivalent equipment, on other days or locations. Parameters such as plate count, tailing factors; resolution and repeatability are determined.

These parameters are studied by analysis of a system suitability sample that is a mixture of main active drug and expected or a known impurity. The reproducibility (%R.S.D.) of five or six replicates is calculated and compared to predetermined specification limits.

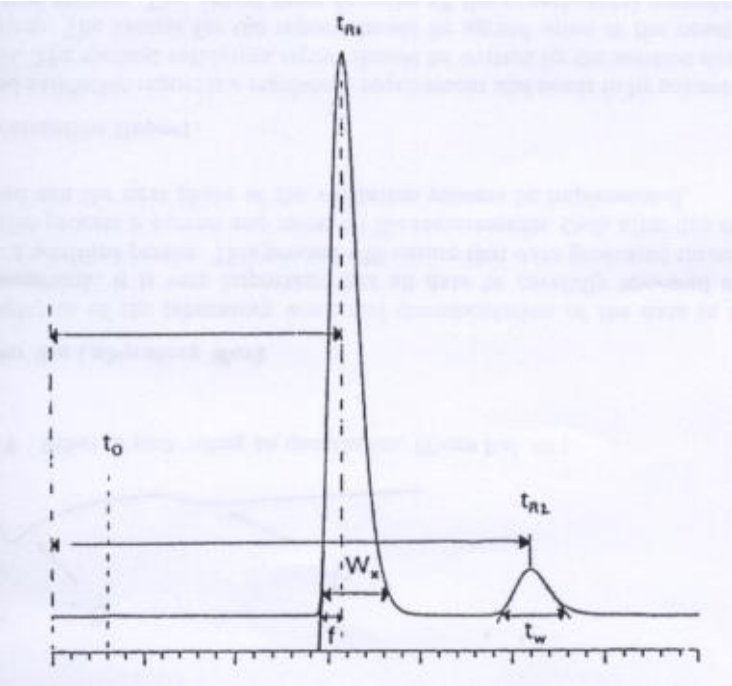


Fig. 1 Definition of terms for system suitability parameters [18]

Where: **W_x** **width of the peak determined at 5% (0.05) from the baseline of the peak height**

f **distance between peak maximum and peak front at Wx**

t_0 **elution time of the void volume or non-retained components**

t_r **retention time of the analyte**

t_w **peak width measured at baseline of the extrapolated straight sides to baseline**

CAPACITY FACTOR

The capacity factor k' is a measure of the degree of retention of a solute (8).

$$k' = \frac{t_R - t_0}{t_0} \quad (8)$$

t_R retention time of the component (min)
(time measured from time of injection to time of elution of peak maximum)

t_0 elution time of the void (dead) volume or non-retained components (min)
(time that the solute needs to be transported with mobile phase until gets elute time)

The peak should be well resolved from other peaks and the void volume, generally k' > 2.0.

NUMBER OF THEORETICAL PLATES

The column efficiency is expressed as a number of theoretical plates *N* (9).

$$N = 16\left(\frac{t_R}{W}\right)^2 = 5.54\left(\frac{t_R}{W_{0.5}}\right)^2 \qquad (9)$$

W width of peak measured at baseline of the extrapolated straight sides to the baseline (min)

$W_{0.5}$ width of peak at half height (min)

The theoretical plate number depends on elution time but in general should be > 2000.

RESOLUSION

Resolution R_s (10) is a measure of how well peaks are separated from each other.

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2} \qquad (10)$$

$t_{1,2}$ retention times of two components (min)

$W_{1,2}$ corresponding widths of the bases of the peaks (min)

Resolution value should be > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.).

TAILING FACTOR

The accuracy of quantitation decreases with increase in peak tailing *T* due to improper peak integration (the area under the peak will not be accurate) (11).

$$T = \frac{W_{0.05}}{2f} \qquad (11)$$

$W_{0.05}$ width of peak at 5% height (min)

f distance between peak maximum and peak front at $W_{0.05}$ (min)

Tailing factor ≤ 2 is required for HPLC study.

REPEATABILITY

Repeatability expressed as %R.S.D. (12) indicates the agreement of results obtained from measurement of five or six replicates of standard solution.

$$R.S.D.(\%) = \frac{100}{\bar{x}} \cdot \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \qquad (12)$$

x_i individual measurement in a set of measurement

$\bar{x}$ arithmetic mean of the set

n number of individual measurements

R.S.D. ≤ 1% for more than 5 measurements is desirable.

RELATIVE RETENTION

Relative retention α is a measure of the relative location of two peaks (13). This is not an essential parameter so long as the resolution is stated.

$$\alpha = \frac{k'_1}{k'_2} = \frac{t_{R1} - t_0}{t_{R2} - t_0} \qquad (13)$$

$k'_{1,2}$ capacity factor of two components peaks

$t_{R1,2}$ retention time of two components

2 AIMS OF THE STUDY

Main aim of the study is quantification of benzocaine and its degradation products (*p*-nitrobenzoic acid ethylester (EPNB) and *p*-aminobenzoic acid (PABA)) in Herplex gel. Method for determination of all three components will be developed following existing literature data [7].

Herplex gel, Galex d.d. containing benzocaine, zinc oxide, white vaseline, liquid paraffin, anhydrous lanolin, purified water, maltodextrin, colloidal anhydrous silicon dioxide and Melissae extractum siccum will be analysed.

HPLC method will be able to test the most important validation parametres. The detection limits and quantification limits will be determined for EPNB and PABA.

HPLC method should allow determining benzocaine, EPNB and PABA with no interference with excipients, quantification of benzocaine to ensure that the concentration of active substance is within the range 90-110% of the nominal concentration, and reliable verification of degradation products presence.

Method will be used also for monitoring of these degradation products during manufacturing process and storage of pharmaceuticals with intention to control the quality (concentration of degradation products). The quantity of active substance in final product is assessed with titration method in the manufacturing process.

3 EXPERIMENTAL

3.1 MATERIALS

3.1.1 Standards

- BENZOCAINE microcrystalline, $C_9H_{11}NO_2$, M = 165.19 g/mol, Galex d.d., Murska Sobota, Slovenia
- *p*-AMINOBENZOIC ACID, $C_7H_7NO_2$, M = 137.136 g/mol, Fluka, Buchs, Sweeden
- *p*-NITROBENZOIC ACID ETHYLESTER, $C_9H_9NO_4$, M = 195.17 g/mol, Fluka, Buchs, Sweeden

3.1.2 Chemicals and reagents

- METHANOL for liquid chromatography, CH_3OH, M = 32.04 g/mol, Merck, Darmstadt, Germany
- ACETIC ACID (glacial) 100 % GR for analysis, CH_3COOH, M = 60.05 g/mol, Merck, Darmstadt, Germany
- TETRAHYDROFURAN for liquid chromatography, C_4H_8O, M = 72.11 g/mol, Merck, Darmstadt, Germany
- DICHLOROMETHANE GR for analysis, CH_2Cl_2, M = 84.93 g/mol, Merck, Darmstadt, Germany
- HPLC grade WATER, H_2O, Katedra za klinično biokemijo, Fakulteta za Farmacijo, Univerza v Ljubljani, Slovenia
- MELISSAE EXTRACTUM siccum, Galex d.d., Murska Soboty, Slovenia
- ACETONE GR for analysis, CH_3COCH_3, M = 58.08 g/mol, Merck, Darmstadt, Germany
- DIMETHYL SULFOXIDE CHROMASOLV® for high-performance liquid chromatography, C_2H_6OS, M = 78.13 g/mol, Sigma-Aldrich, Steinheim, Germany

- BENZYL ALCOHOL GR for analysis, $C_6H_5CH_2OH$, M = 108.14 g/mol, Merck, Darmstadt, Germany
- 1-PROPANOL for liquid chromatography, $CH_3CH_2CH_2OH$, M = 60.10 g/mol, Merck, Darmstadt, Germany
- 2-PROPANOL gradient grade for liquid chromatography, $CH_3CH(OH)CH_3$, M = 60.10 g/mol, Merck, Darmstadt, Germany

3.1.3 Pharmaceutical products for determination

- HERPLEX ointment, 15 g, Galex d.d., Murska Sobota, Slovenia

Herplex gel (15 g) contains 5% of benzocaine (50 mg/g of gel) (*Benzocainum*), Melissae extractum siccum (*Melisa officinalis*), white vaseline (*Vaselinum album*), liquid paraffin (*Paraffinum liquidum*), anhydrous lanolin (*Adeps lanae*), purified water (*Aqua purificata*), maltodextrin, colloidal anhydrous silicon dioxide and zinc oxide (100 mg/g of gel) (*Zinci oxidum*).

Batches analysed: No 07670904 (expiration 09/2005)
No 09341105 (expiration 11/2006)
No 02870306 (expiration 03/2007)
No 05990706 (expiration 07/2007)
No 06000706 (expiration 07/2007)
No 06010706 (expiration 07/2007)
No 09771206 (expiration 12/2007) – three sample (1, 2, 3)
No 09781206 (expiration 12/2007) – three sample (1, 2, 3)

- HERPLEX without benzocaine, Galex d.d., Murska Sobota, Slovenia

Herplex excipient consists of Melissae extractum siccum (*Melisa officinalis*), white vaseline (*Vaselinum album*), liquid paraffin (*Paraffinum liquidum*), anhydrous lanolin (*Adeps lanae*), purified water (*Aqua purificata*), maltodextrin, colloidal anhydrous silicon dioxide and zinc oxide (*Zinci oxidum*).

Batches analysed: No 02880306
No 011206
No 111206

3.2 EQUIPMENT

- Analytical balance AG 245, Mettler Toledo, Columbus, USA
- Water Polishing System PURELAB® Classic UF, ELGA, UK
- Ultrasonic cleaner SONIS, Iskra, Sentjernej, Slovenia
- UV-Visible Spectrophotometer 8453 Hewlett Packard, Agilent Technologies, USA
- Vacuum filtration unit, Sartorius AG, Goettingen, Germany
- Cellulose acetate filter, 0.45 μm, Sartorius AG, Goettingen, Germany
- Minisart RC 25 filter, 0.45 μm, Sartorius AG, Goettingen, Germany
- Filter Paper 388, Sartorius AG, Goettingen, Germany
- Columns:
  - Eclipse XDB C18 (150 x 4.6 mm, 5 μm), No 993067-902, Agilent Technologies, USA
  - Nucleosil 100 C18 (150 x 6.6 mm, 5 μm), No 049891, Agilent Technologies, USA
- HPLC system Agilent 1100 Series, Agilent Technologies, USA:
  - Quaternary pump G1312A
  - Automatic injector
  - Degasser G1379A
  - Column oven G1316A
  - DAD detector G1315A
  - Software package ChemStation for LC3D, Rev. B.01.01 [164] SR1, 2005, Agilent Technologies, USA

3.3 CHROMATOGRAPHIC CONDITIONS

Two methods for determination of benzocaine and its degradation products were developed. The method A was used for determination and quantification of benzocaine and EPNB and the second method, the method B, was used for determination and quantification of PABA.

3.3.1 Chromatographic method A

Column: Eclipse XDB C18, 150 x 4.6 mm, 5 µm particle size

Mobile phase: glacial acetic acid solution (10%, v/v) and methanol in a gradient programme (Table 3)

Injection volume: 10 µm

Flow rate: 2.0 ml/min

Temperature: 40 °C

DAD detector: 258 nm

Determination time: 30 min

Table 3

Gradient profile program of method A for benzocaine and EPNB

Time (min)	Acetic acid glacial (%)	Methanol (%)
0	90	10
9	90	10
13	60	40
25	60	40
28	90	10
30	90	10

3.3.2 Chromatographic method B

Column: Eclipse XDB C18, 150 x 4.6 mm, 5 µm particle size

Mobile phase: glacial acetic acid solution (10%, v/v) and methanol (90% / 10%)

Injection volume: 10 µm

Flow rate: 1.0 ml/min

Temperature: 40 °C

DAD detector: 258 nm

Determination time: 10 min

3.4 PREPARATION OF SOLUTIONS

3.4.1 Preparation of solutions for UV spectra measurement

Solutions for UV detection were prepared separately for each active. The weighed amount of individual active (5.78 mg of benzocaine, 5.21 mg of EPNB and 4.48 mg of PABA) was diluted with tetrahydrofuran in a 50 ml volumetric flask. 1 ml of the solution was transferred to a 10 ml volumetric flask and it was diluted with THF. After that, 5 ml of the solution was placed in a 10 ml volumetric flask and diluted with THF. PABA solution had to be diluted again therefore 5 ml of solution was transferred to a 10 ml volumetric flask and diluted with THF. The final concentration of each active (5.78 μg/ml for benzocaine, 5.21 μg/ml for EPNB and 2.24 μg/ml for PABA) gave the absorbance below 1.0 on a spectrophotometer.

3.4.2 Preparation of mobile phase

The mobile phase for both methods consists of glacial acetic acid solution (10%, v/v) and methanol. Proportion of constituents of mobile phase is influenced by type of used method. In method A constituents were carried as a gradient programme (Table 3) whereas in method B proportion of compositions is constant, 90% solution of acetic acid glacial and 10% of methanol.

Glacial acetic acid solution was prepared by diluting of acetic acid glacial 100% with HPLC grade water which was filtrated through a 0.45 μm Cellulose acetate filter or filter paper 388.

3.4.3 Preparation of standard solutions

a) STANDARD SOLUTIONS FOR LINEARITY DETERMINATION

Working standard solutions of benzocaine and its two degradation products were prepared by dissolving approximately 20 mg of individual active (true amounts are listed below) with tetrahydrofuran in 100 ml volumetric flask. Based on this solution

and with adequate dilution with glacial acetic acid solution (Table 4 and Table 5), 6 standard solutions for determination of benzocaine linearity and 10 standard solutions for EPNB and PABA were prepared.

For benzocaine linearity we weighed 20.20 mg (samples No 1.1 and No 1.2) and 20.20 mg (samples No 2.1 and No 2.2) of benzocaine, 20.04 mg of EPNB (samples No 1.1 and No 1.2) and 20.00 mg of EPNB (samples No 2.1 and No 2.2) and 20.10 mg of PABA (samples No 1.1 and No 1.2) and 20.00 mg of PABA (samples No 2.1 and No 2.2). Two working standard solutions were prepared.

Table 4

Composition of standard solutions for linearity study of benzocaine

Solution No	Concentration (µg/ml)	Composition of solutions (100 ml)
1.	200	20 mg benzocaine + THF
2.	160	80 ml of sol. No 1. + acetic ac. gl. (10%)
3.	80	50 ml of sol. No 2. + acetic ac. gl. (10%)
4.	40	50 ml of sol. No 3. + acetic ac. gl. (10%)
5.	16	40 ml of sol. No 4. + acetic ac. gl. (10%)
6.	8	50 ml of sol. No 5. + acetic ac. gl. (10%)

Table 5

Composition of standard solutions for linearity study of degradation products (EPNB and PABA)

Solution No	Concentration (µg/ml)	Composition of solutions	Total volume (ml)
1.	200	20 mg EPNB (PABA) + THF	100
2.	160	80 ml of sol. No 1. + acetic ac. gl. (10%)	100
3.	80	50 ml of sol. No 2. + acetic ac. gl. (10%)	100
4.	40	50 ml of sol. No 3. + acetic ac. gl. (10%)	100
5.	16	40 ml of sol. No 4. + acetic ac. gl. (10%)	100
6.	8	50 ml of sol. No 5. + acetic ac. gl. (10%)	100
7.	4	25 ml of sol. No 6. + acetic ac. gl. (10%)	50
8.	2	25 ml of sol. No 7. + acetic ac. gl. (10%)	50
9.	1	25 ml of sol. No 8. + acetic ac. gl. (10%)	50
10.	0.5	25 ml of sol. No 9. + acetic ac. gl. (10%)	50

b) MIXING OF EXCIPIENT WITH THE ACTIVE

Approximately 9.5 g of Herplex excipient was weighed and placed into mortar containing 0.5 g of active (as amount of benzocaine in Herplex gel) and mixed with pestle. Extraction procedures of active are explained below (method A and B).

Mixtures one of the active (benzocaine, EPNB or PABA) and Herplex excipient were prepared and analysed. Precision and accuracy studies of all substances were carried out with these mixtures.

Exact amount of individual active, amount of Herplex excipient mixed with individual active (total amount) and amount of mixture used for particular tests are listed in Table 6.

Table 6
Composition of mixtures of active and excipient for precision and accuracy detection

Active	Amount of active (mg)	Total amount (mg)	Amount used for test (mg)
Benzocaine	501,30	10 040,1	1 126,90
	502,10	10 118,0	969,00
	501,30	10 103,3	1 063,50
EPNB	500,00	10 038,8	1 130,00
	501,70	10 260,7	1 220,90
	501,80	10 164,0	983,50
PABA	499,40	10 009,6	1 079,90
	500,60	9 997,8	1 125,60
	501,30	10 025,1	1 058,80

Evaluation of method specificity was made on samples of Herplex excipients of different age according to method A and B.

RECOVERY OF BENZOCAINE AND EPNB (METHOD A)

Approximately 1 g of mixture of either Herplex excipient and benzocaine or Herplex excipient and EPNB (which corresponds to 50 mg of active substance content in 1 g

of gel) was weighed, placed into a 50.0 ml volumetric flask and was made up to the volume with dichloromethane. This mixture was sonicated for 5 min. An appropriate amount of solution was filtered through the Minisart RC 25 filter (0.45 μm) and parallel samples of 2 ml of solution were prepared. After that, the samples were evaporated on a water bath at temperature 50-55 °C. To the rests 5 ml of tetrahydrofuran were added. The solutions were again sonicated; 3 ml of solutions were transferred into 10 ml volumetric flasks and diluted with glacial acetic acid solution (10%, v/v). These solutions were filtered through the Minisart RC 25 filter (0.45 μm) and analysed with HPLC.

RECOVERY OF PABA (METHOD B)

For study of PABA, approximately 1 g of Herplex excipient and PABA mixture was weighed, placed into a 50.0 ml volumetric flask and tetrahydrofuran was added. This mixture was sonicated for 5 min. Afterwards an appropriate amount of solution was filtered through the Minisart RC 25 filter (0.45 μm). Parallel samples of 2 ml of solution were transferred into 25 ml volumetric flasks and diluted with solution of glacial acetic acid (10%, v/v). These solutions were filtered through the Minisart RC 25 filter (0.45 μm) and analysed with HPLC.

3.4.4 Preparation of assay samples

EXTRACTION OF BENZOCAINE AND EPNB FROM GEL (METHOD A)

For extraction of benzocaine and EPNB, approximately 1 g of topical gel (which corresponds to 50 mg of benzocaine) was weighed, placed into a 50.0 ml volumetric flask and was made up to the volume with dichloromethane. This mixture was sonicated for 5 min. Afterwards an appropriate amount of solution was filtered through the Minisart RC 25 filter (0.45 μm). Parallel samples of 2 ml of solution were taken and evaporated on a water bath at temperature 50-55 °C. To the rests 5 ml of tetrahydrofuran were added. The solutions were again sonicated, 3 ml of solutions were transferred into 10 ml volumetric flasks and diluted with glacial acetic acid solution (10%, v/v). These solutions were filtered through the Minisart RC 25 filter (0.45 μm) and analysed with HPLC.

EXTRACTION OF PABA FROM GEL (METHOD B)

For extraction of PABA, approximately 1 g of topical gel was weighed, placed in a 50.0 ml volumetric flask and diluted with tetrahydrofuran. This mixture was sonicated for 5 min. and an appropriate amount of solution was filtered through a Minisart RC 25 filter (0.45 μm). Parallel samples of 2 ml of solution were prepared and transferred into 25 ml volumetric flasks. After that, the solutions were diluted with glacial acetic acid solution (10%, v/v). These solutions were filtered through the Minisart RC 25 filter (0.45 μm) and analysed with HPLC.

Exact amounts of Herplex gels used for assay determination are presented in

Table 7. For determination were used Herplex gels of all batches mentioned in chapter 3.1.3.

Amounts of Herplex gels (batch No 09771206, sample 1 and 3) used for thermal stability determination are presented in Table 8. The samples (E and F) were prepared according the extraction processes (method A and B) described above

Table 7

Herplex gel´s amounts of all batches taken for performing of assay study

Herplex gel	Sample	Amount of gel (mg)	
		Method A	Method B
A	A1	1 177,50	1 025,50
No 07670904	A2	1 164,50	1 068,00
B	B1	1 018,70	1 048,50
No 02870306	B2	1 091,90	1 087,10
C	C1	1 001,80	1 152,30
No 09341105	C2	1 051,60	1 053,90
D	D1	1 040,91	1 068,20
No 06010706	D2	1 003,50	1 050,80
E	E1	992,00	1 030,00
No 09771206-1	E2	1 063,40	1 137,70
F	F1	1 007,10	1 167,80
No 09771206-3	F2	1 041,50	-
G	G1	989,00	1 059,10
No 09781206-1	G2	931,00	998,90
H	H1	1 210,90	1 092,40
No 09781206-3	H2	1 140,80	1 111,40
I	I1	972,20	917,71
No 05990706	I2	1 020,60	-

J	J1	991,60	1 023,33
No 06000706	J2	1 037,70	-
K	K1	1 116,30	1 038,46
No 06010706	K2	1 120,70	-

Table 8

Herplex gel´s amounts (batch No 09771206) taken for thermal stability study

Herplex gel	Sample	Amount of gel (mg)	
		Method A	Method B
E No 09771206-1	E1	1 139,70	1 092,20
F No 09771206-3	F1	917,70	942,20

3.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.5.1 Development of HPLC conditions

Chromatography conditions were set up in accordance with the Pérez-Lozano et al. [7] method. Two analytical columns were tested. The column Nucleosil 100 C18 (150 x 6.6 mm, 5 μm) was not successful in flow rate 2 ml/min required for detection whereas the column Eclipse XDB C18 (150 x 4.6 mm, 5 μm) allowed detection under this condition.

Chromatogram on

Fig. 2 indicates Herplex excipient mixed with benzocaine, PABA and EPNB obtained by method A (chapter 3.3.1). PABA has retention time of 1.095 min and its peak interferes with another peak of mobile phase. Therefore another method, method B, (chapter 3.3.2) established for determination of PABA has the flow rate 1 ml/min. PABA elutes at 2.193 min and another peak in the vicinity of PABA peak does not appeared (

Fig. 3).

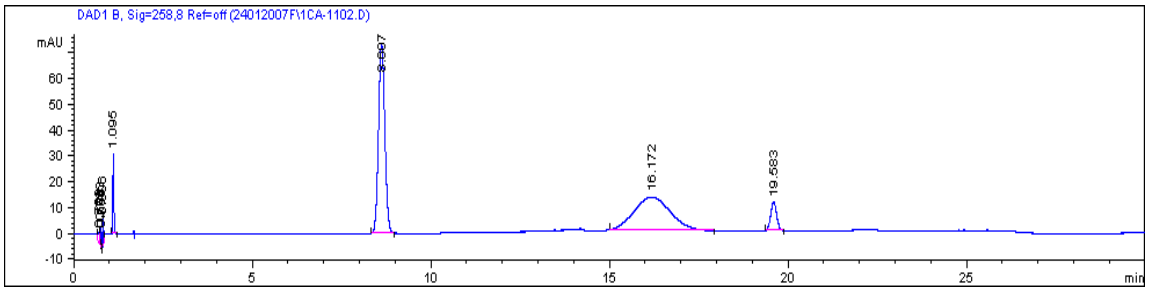


Fig. 2 Chromatogram of Herplex excipient mixed with benzocaine, PABA and EPNB analysed by method A (PABA elutes at 1.095, benzocaine at 8.697 and EPNB at 19.583 min). The peak (retention time 16.172 min) comes from the filter paper 388.

The peak with retention time 16.172 min is from the filter paper 388 as is explained later in the chapter 4.2.1.

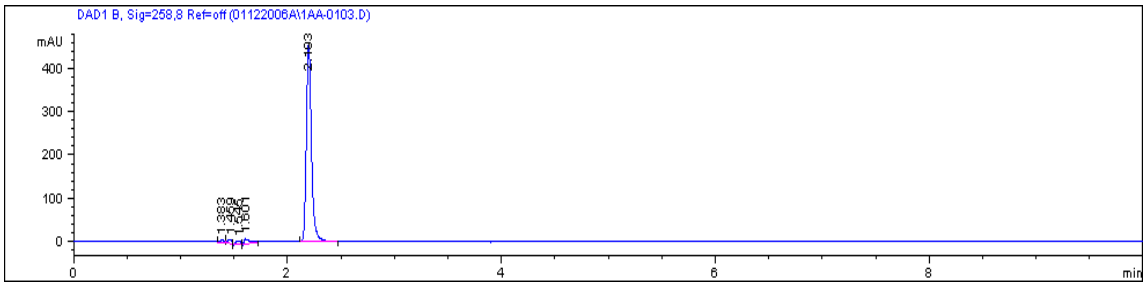


Fig. 3 Chromatogram of Herplex excipient mixed with PABA analysed by method B (PABA elutes at 2.193 min).

Determination and quantification of benzocaine and its degradation products were performed by two methods. Method A was used for benzocaine and EPNB determination and method B for PABA determination (as described in chapter 3.4.4).

The extraction effectivity of both methods was evaluated by comparison of results obtained from extraction of benzocaine and EPNB by method B and extraction of PABA by method A with results obtained during validation.

Extraction of PABA by method A was insufficient already during development of isolation procedure (chapter 3.5.2) therefore this extraction effectivity was not

analysed again. Method B extraction was verified on Herplex gel samples and EPNB recovery samples. The results obtained are shown in

Table 9 and Table 10.

Benzocaine extractions from Herplex gels are presented in

Table 9. Samples for extraction were prepared according to procedure of method B (chapter 3.4.4).

Table 9

% of benzocaine recovery gained from Herplex gels (batches A-C: No 07670904, No 09052006 and No 09341105) according to extraction procedure of method B

Sample	Theoretical c of BZC (µg/ml)	Vial No	Mean of peak areas (mAU*s)	Measured c of BZC (µg/ml)	% recovery
A	92,19	1	708,76	89,10	96,64
		2	718,50	90,31	97,95
B	88,78	1	698,69	87,85	98,95
		2	715,35	89,92	101,28
C	95,74	1	735,38	92,40	96,51
		2	741,29	93,14	97,28

By extraction of benzocaine with method B, 98.10% of recovery was gained (it responds to the guidelines required value 100 ± 5%).

Method B was also examined on mixtures of Herplex excipient and EPNB prepared for EPNB accuracy study (Table 10). Mean value of EPNB recovery obtained was 90.91%, which is below 100 ± 5%, and it does not respond to recommended values. Therefore method A was used for determination and quantification of benzocaine and EPNB.

Table 10

Results of EPNB recovery method obtained according to extraction procedure of method B

No	Vial	Added c (µg/ml)	Found c (µg/ml)	Recovery (%)	R.S.D. (%)
1	1.1.	79,06	70,84	89,60	0,1771
2	1.2.		74,14	93,78	0,2244
3	2.1.	79,06	70,34	88,97	0,1523
4	2.2.		73,48	92,94	0,1272
5	3.1.	79,06	70,06	88,62	0,2009
6	3.2.		72,37	91,54	0,3766

3.5.2 Development of isolation procedure for benzocaine, EPNB and PABA from gel

For establishment of this method many analytical procedures (solvents and proportions of dilution) were tested. Pérez-Lozano et al. [7] and their validated method has used ethanol and adequate dilution for extraction from bioadhesive gel.

Different extraction media were used for development of method – acetone, methanol (MeOH), ethanol, 1-propanol, 2-propanol, benzyl alcohol, dimethylsulfoxide, tetrahydrofuran (THF) and dichloromethane (DCHM). Only last two THF and DCHM were effective.

Series of dilutions and measurements (with chromatographic conditions of method A) followed. In all cases 50 ml volumetric flask (VF) containing 1 g of mixture (generated from 9.5 g of Herplex excipient and 0.5 g of benzocaine) was made up to the volume with one of solvents listed below, sonicated and filtered through Minisart RC 25 filter (0.45 μm).

Solvents and proportions of dilution, which were analysed:

1) **Solution of tetrahydrofuran** in 50 ml volumetric flask and 1 g of mixture.
   Additional dilution:
   a) 10 ml of solution and glacial acetic acid solution in 500 ml VF
   b) 5 ml of solution and glacial acetic acid solution in 10 ml VF
      In both solutions with glacial acetic acid solution precipitation occurred.
   c) 5 ml of solution and THF in 50 ml VF
   d) 2 ml of solution and THF in 50 ml VF
      On chromatograms were non-symmetric peaks.
   e) 5 ml of solution and MeOH in 10 ml VF
   f) 5 ml of solution and MeOH in 50ml VF
   g) 5 ml of solution and DCHM in 10 ml VF
   h) 5 ml of solution and DCHM in 25 ml VF
   i) 5 ml of solution and DCHM in 50 ml VF
      Procedures gave benzocaine recovery about 110%.
2) **Solution of dichloromethane** in 50 ml volumetric flask and 1 g of mixture.
   Additional dilution:
   a) 5 ml of solution and DCHM in 5 VF
   b) 5 ml of solution and DCHM in 20 VF
   c) 5 ml of solution and DCHM in 50 VF
      Procedures gave benzocaine recovery from 100 - 110%.

Since DCHM and acetic acid solution are not miscible next procedures were found on evaporation of DCHM.

d) 2 ml (also 1 ml) of solution was evaporated and 2 ml of THF was added, then 1 ml was transferred in 10 ml VF and diluted with THF

Peak presented on chromatogram was non-symmetric.

e) 2 ml of solution was evaporated and 2 ml THF was added, then 1 ml was transferred in 10 ml VF and diluted with acetic acid solution

f) 2 ml of solution was evaporated and 10 ml THF was added, then 5 ml was transferred in 10 ml VF and diluted with MeOH

g) 2 ml of solution was evaporated and 10 ml THF was added, then 5 ml was transferred in 10 ml VF and diluted with acetic acid solution

These samples gave benzocaine recovery in a range 102 - 105% (with MeOH approximately 105%).

h) 2 ml of solution was evaporated and 5 ml THF was added, then 3 ml was transferred in 10 ml VF and made up with acetic acid solution

99.5-103.0% recovery of benzocaine was gained.

Values of benzocaine recovery 100 ± 5% are considered as sufficient. The procedure h) was proved on mixture EPNB or PABA with Herplex excipient and recoveries obtained were in range 95 - 98% for EPNB and 90 - 93% for PABA. That is why another method had to be established for extraction of PABA from Herplex gel.

3) **Solution of dichloromethane** in 50 ml volumetric flask and 1 g of mixture were sonicated and filtered. Afterwards:

a) 2 ml of solution was evaporated and 10 ml THF was added. 5 ml was transferred in 10 ml VF and made up with acetic acid solution

b) 2 ml of solution was evaporated and 10 ml THF was added. 8 ml was transferred in 10 ml VF and made up with acetic acid solution

PABA recovery were approximately 95%

4) **Solution of tetrahydrofuran** in 50 ml volumetric flask and 1 g of mixture were sonicated and filtered. Afterwards:

a) 2 ml of solution was diluted in 25 ml VF with acetic acid solution.

PABA recovery was approximately 100%.

3.5.3 Validation of HPLC method

SPECIFICITY

For specificity study samples of placebo (Herplex excipient), Herplex excipient mixed with benzocaine (in concentration which corresponds to Herplex gel) and EPNB (in concentration near the concentration of LOQ) were prepared for method A. Samples of Herplex excipient, Herplex excipient mixed with PABA (in concentration near the concentration of LOQ) were prepared for method B. Each sample was prepared throughout the processes described in a part 3.4.3. Chromatograms of Herplex gel obtained by method A and B are attached to in specificity study.

LINEARITY

Linearity study was performed by measuring of 6 concentration levels (standard solutions) of benzocaine (8, 16, 40, 80, 160 and 200 μg/ml) and 10 concentration levels (standard solutions) of EPNB and PABA (0.5, 1, 2, 4, 8, 16, 40, 80, 160 and 200 μg/ml) prepared according to procedure and in amounts described in chapter 3.4.3. Benzocaine linearity and EPNB linearity were carried out using method A and PABA linearity was carried out using method B.

Analysis was performed with two pairs of solution (chapter 3.4.3, No 1.1, 1.2 and 2.1, 2.2) with triplicate injection (4 sets of six, ten concentrations in triplicate respectively) for benzocaine, EPNB and PABA individually.

Calibration curve, linear regression equation (y-intercept and slope of the regression line) and correlation coefficient were calculated.

PRECISION

Precision was proved on the samples of Herplex excipient and individual active mixtures prepared according to processes (method A and B) described in chapter 3.4.3. Each active (benzocaine, EPNB and PABA) was prepared in triplicate (3 mixtures of benzocaine (1, 2 and 3), EPNB and PABA respectively). From each mixture 2 samples were prepared (from mixture 1 vials 1.1, 1.2; from mixture 2 vials 2.1, 2.2 and from mixture 3 vials 3.1, 3.2). Every sample was injected and measured three times.

Outcomes of each substance were reported as SD (formula 3) and %R.S.D (formula 4) of six samples of 100% concentration, each three times injected.

ACCURACY

For accuracy determination the same samples as for precision were used, prepared in agreement with chapter 3.4.3. (amounts and procedures). Each active (benzocaine, EPNB and PABA) was prepared in triplicate (3 mixtures of benzocaine (1, 2 and 3), EPNB and PABA respectively). From each mixture 2 samples were prepared (from mixture 1 vials 1.1, 1.2; from mixture 2 vials 2.1, 2.2 and from mixture 3 vials 3.1, 3.2). Every sample was injected and measured three times.

Method accuracy for benzocaine and its degradation products were evaluated as % of recovery (formula 2) and %R.S.D (formula 4). of six samples of 100% concentration, each three times injected.

LOQ and LOD

These limits were determined based on the S.D. of the y-intercepts of regression line and the slope from calibration curve of the mean values (equation 6 and 7) of EPNB and PABA.

4 RESULTS

4.1 UV SPECTRA DETECTION

UV spectra of studied actives were measured to prove that the absorption wavelength 258 nm used in HPLC method was convenient and effective for quantification of benzocaine and its degradation products.

Tetrahydrofuran was employed as a blank sample in all cases and solutions of individual compounds were prepared according to procedures stated in chapter 3.4.1.

4.1.1 Benzocaine

Maximum of absorption wavelength obtained for benzocaine were 220.0 and 289.0 nm (Fig. 4).

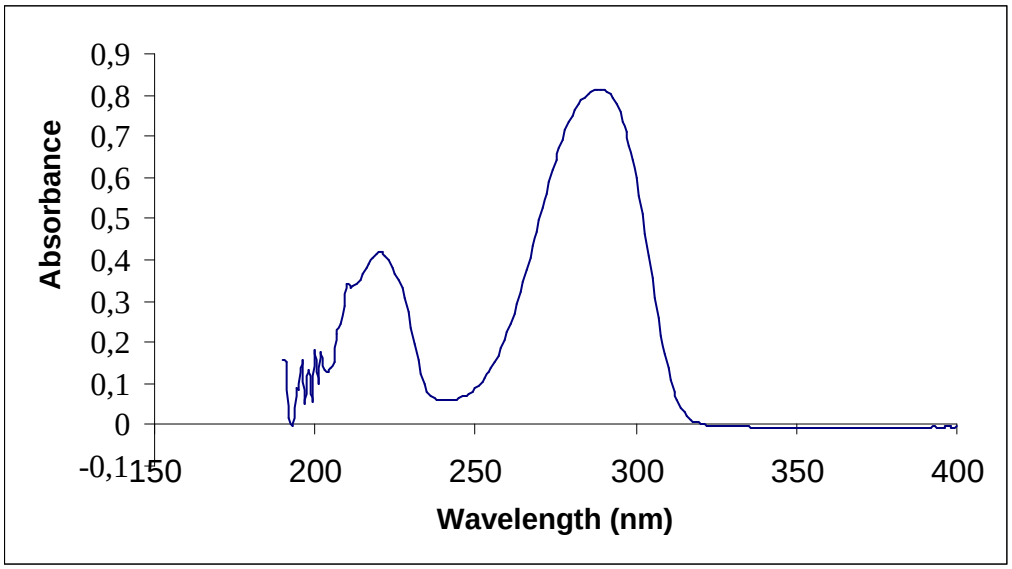


Fig. 4 UV spectra of benzocaine solution with concentration 5.78 µg/ml

4.1.2 *p*-Nitrobenzoic acid ethylester

Maximum of absorption wavelength obtained for EPNB were 210.0 and 260.0 nm (Fig. 5).

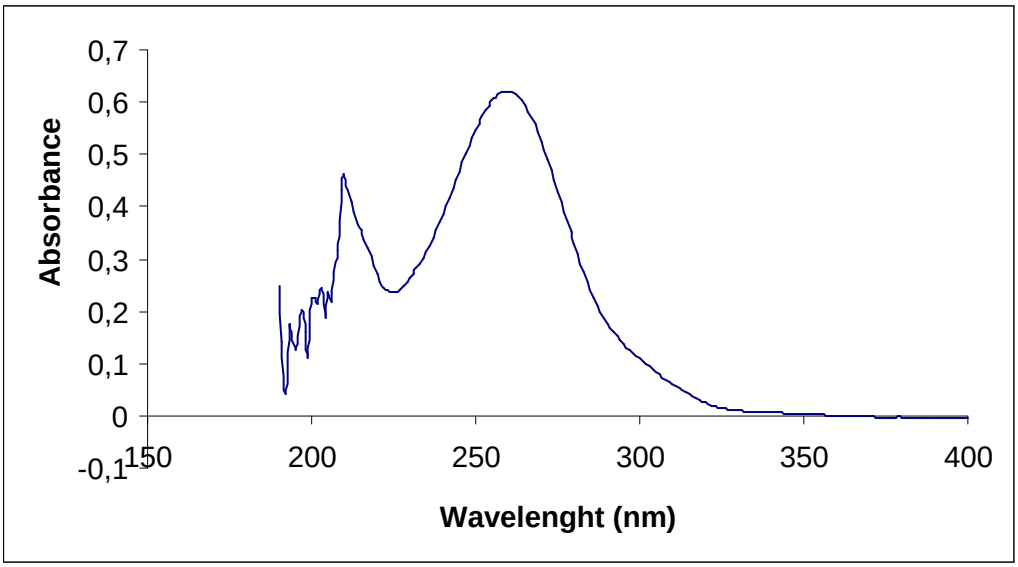


Fig. 5 UV spectra of EPNB solution with concentration 5.21 µg/ml

4.1.3 *p*-Aminobenzoic acid

Maximum of absorption wavelength obtained for PABA were 221.0 and 284.0 nm (Fig. 6).

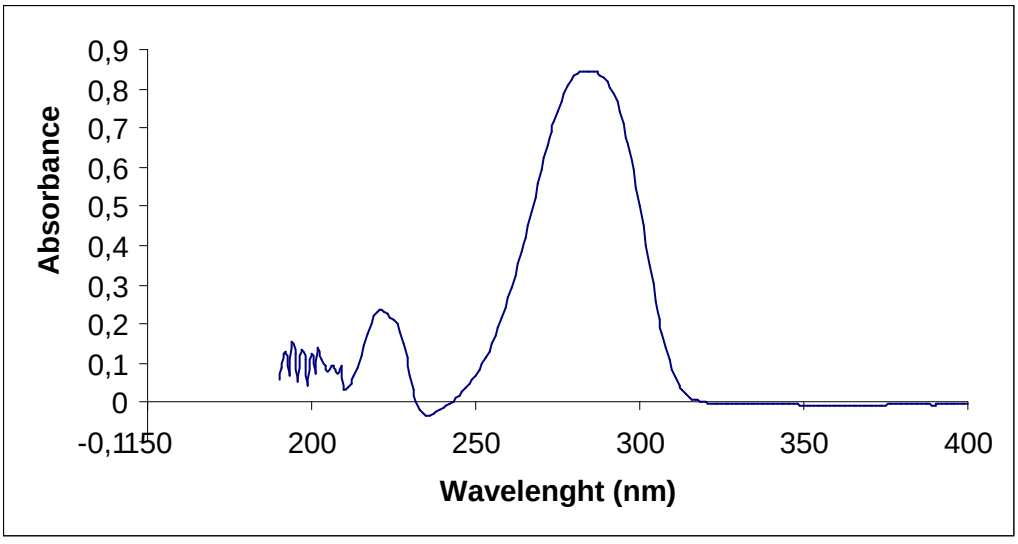


Fig. 6 UV spectra of PABA solution with concentration 2.24 µg/ml

Although the wavelength of maximum absorbance for benzocaine is 289 nm, the used wavelength 258 nm chosen following the validated method of benzocaine analysed by Pérez-Lozano et al. [7]., is sensitive and acceptable for the determination

of benzocaine. The wavelength 258 nm corresponds to the maximum absorbance for EPNB (260 nm).

4.2 VALIDATION OF ANALYTICAL METHODS

4.2.1 Benzocaine

SPECIFICITY

Method specificity for benzocaine determination was verified by comparison of chromatograms of Herplex excipient, Herplex excipient mixed with benzocaine, Herplex excipient mixed with benzocaine and EPNB and chromatogram of Herplex gel performed by method A.

Chromatogram on
Fig. 7 was provided from Herplex excipient analysed by method A with acetic acid solution in mobile phase filtered through Cellulose acetate filter (0.45 μm).

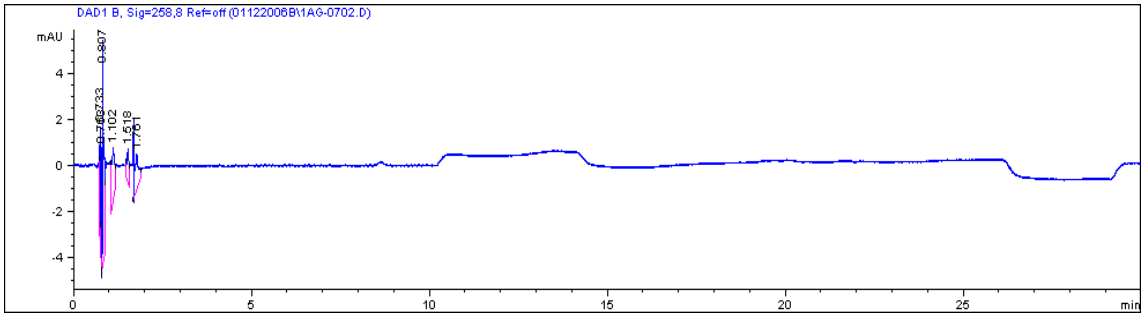


Fig. 7 Chromatogram of Herplex excipient obtained by method A (using Cellulose acetate filter)

The Herplex excipient chromatogram (
Fig. 8) shows an unknown product (peak), which appeared in Herplex excipients and in Herplex gels of all used batches determined by method A. The product has come from filter paper 388 used for filtration of glacial acetic acid solution in mobile phase. Retention time of this product is in range of 15.320 – 17.043 min. and it was found in Herplex gel extracted with method B and analysed with conditions of method A.

For this reason we changed filter and used Cellulose acetate filter (0.45 μm).

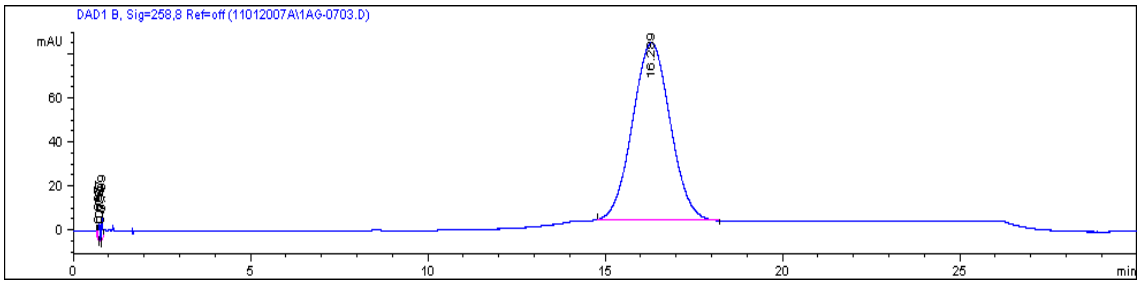


Fig. 8 Chromatogram of Herplex excipient obtained by method A. Peak has come from filter paper 388 used for filtration of glacial acetic acid solution.

Peak appearing at 15.9 min was suspected to come from filter paper 388 (

Fig. 9). Change of filter paper and using of Cellulose acetate filter confirmed this hypothesis since in this case no peak was 15.9 min.

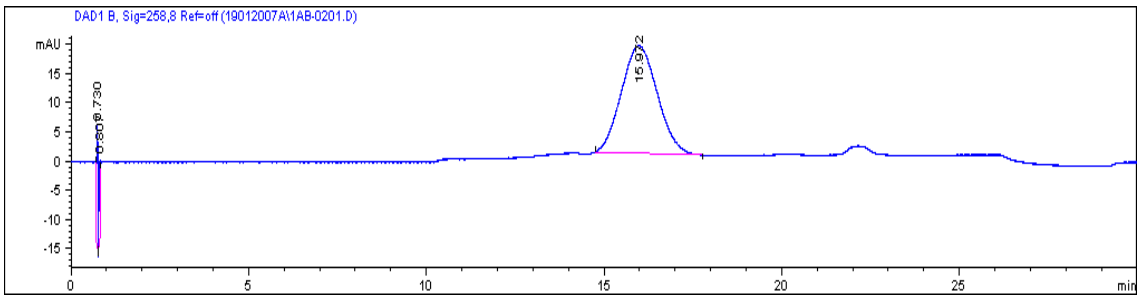


Fig. 9 Chromatogram of HPLC grade water analysed according to conditions of method A (peak of product from filter paper 388)

Benzocaine elutes at 8.594 min. (

Fig. 10). It was checked that benzocaine added to excipient as well as benzocaine in Herplex gel (

Fig. 11) elute at the same retention time. No interfering peaks in the closeness of the peak of benzocaine were observed in sample chromatogram.

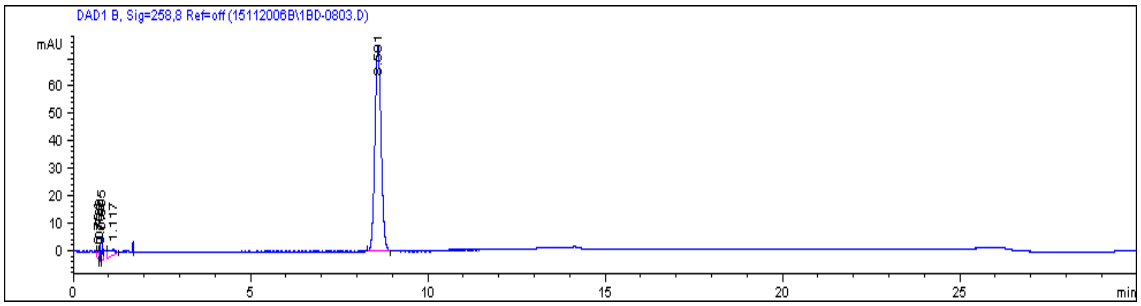


Fig. 10 Chromatogram of Herplex excipient mixed with benzocaine prepared for precision and accuracy study using Cellulose acetate filter, method A

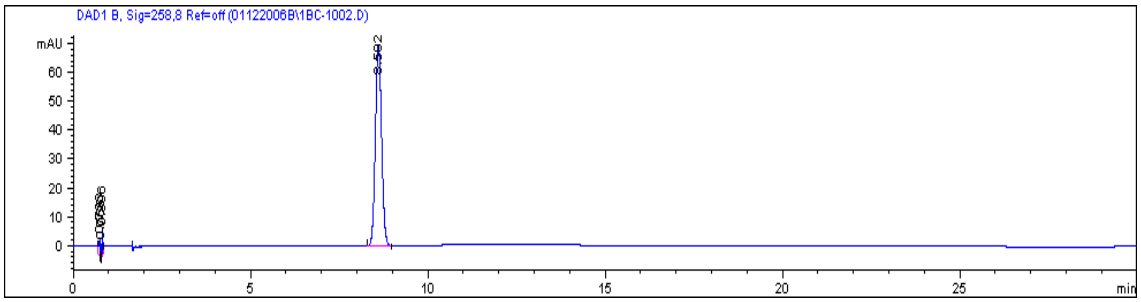


Fig. 11 Chromatogram of Herplex gel obtained by method A using Cellulose acetate filter for filtration of glacial acetic acid solution

Chromatograms on

Fig. 12 and

Fig. 13 show that there were no interference between the excipient and the active and product.

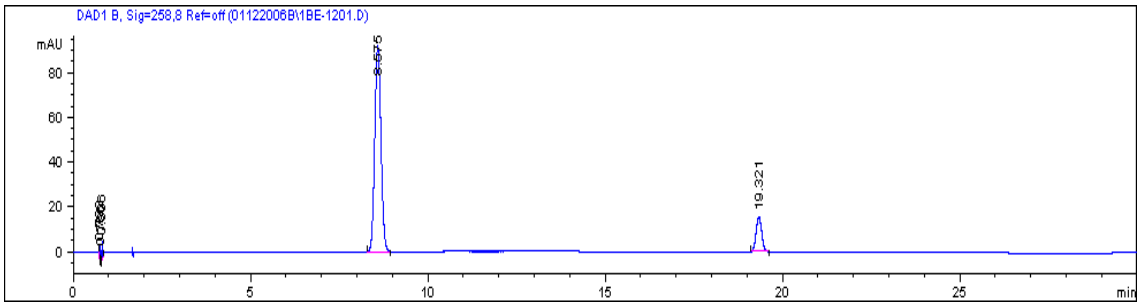


Fig. 12 Chromatogram of Herplex excipient mixed with benzocaine and EPNB obtained by method A, acetic acid for mobile phase was filtered through Cellulose acetate filter

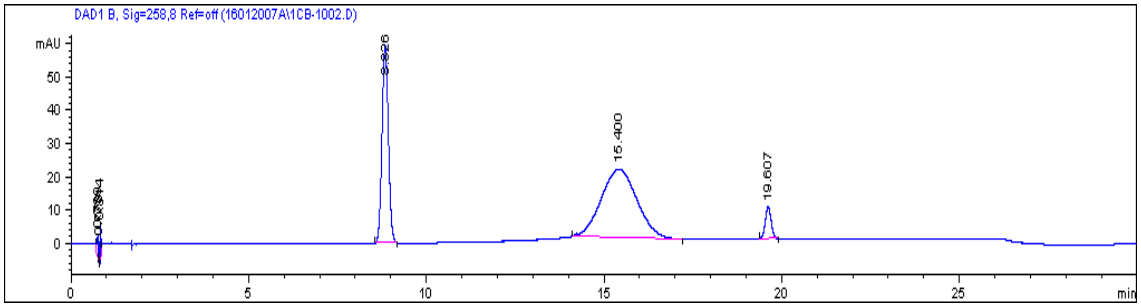


Fig. 13 Chromatogram of Herplex excipient mixed with benzocaine and EPNB analysed by method A with acetic acid solution filtered through filter paper 388 (retention time: benzocaine 8.825, filter paper product 15.400 and EPNB 19.607 min)

LINEARITY

Linear relationship was determined between the peak area of benzocaine and the corresponding concentrations, as shown calibration curve (

Fig. 14) of mean peak areas values from obtained results (Table 11). Measured values of peak areas were adjusted according to concentrations stated in table.

Table 11

Values of peak areas and corresponding concentrations for benzocaine linearity; solution No 1 (benzocaine weigh 20.20 mg) measured two times (sample No 1.1, 1.2), solution No 2 (benzocaine weigh 20.20 mg) measured two times (sample No 2.1, 2.2).

Concentration (µg/ml)	Sample No/Peak area (mAU*s)				Mean (mAU*s)
	1.1.	1.2.	2.1.	2.2.	
8,00	57,27	56,36	57,45	57,26	57,09
16,00	125,06	122,96	125,06	125,06	124,53
40,00	312,20	308,66	312,54	311,19	311,15
80,00	630,08	626,37	633,90	627,45	629,45

160,00	1 275,42	1 263,73	1 300,13	1 291,41	1 282,67
200,00	1 606,26	1 574,70	-	-	-

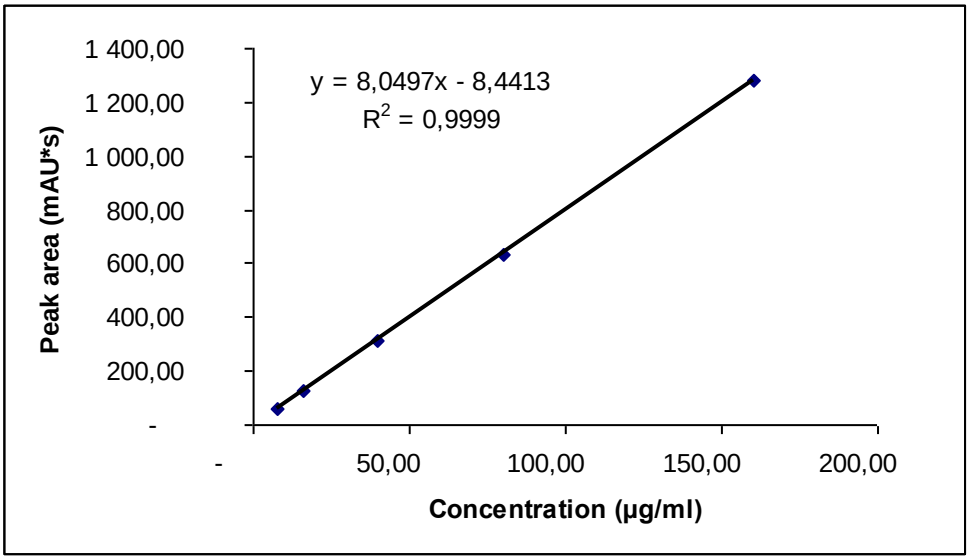


Fig. 14 Calibration curve of benzocaine given from mean values of peak areas obtained by measuring.

The linear regression equation $y = 8.0497x - 8.4413$ and the correlation coefficient R^2 0.9999 were also calculated from mean values. The correlation coefficient of mean values (

Fig. 14) as well as each of the correlation coefficients (Table 12) correspond to the recommended value $R^2 \geq 0.999$.

In Table 12 regression equations, y-intercepts and slopes of regression line for each set of standard samples are reported.

Table 12

Equation of each individual measuring of benzocaine linearity

Sample	Equation for calibration curve	R^2
1.1.	y = 8.0481x - 8.5184	1,0000
1.2.	y = 7.9026x - 4.2264	1,0000
2.1.	y = 8.1453x - 8.4737	0,9998
2.2.	y = 8.0596x - 4.9476	0,9997

RANGE

The method was found to be linear in the range 8.0 – 160.0 μg/ml (
Fig. 14). These concentrations cover area of approximately 10 - 130% of the test concentration of benzocaine.

The concentration 200.0 μg/ml of benzocaine could not be included to the range and calculating since the shape of its chromatographic peak was no symmetric.

PRECISION

Method precision or repeatability was studied at 100% concentration of benzocaine with six samples and triplicate injection of each sample. The results obtained are presented in Table 13 and were calculated according to equation of calibration curve generated from mean values (
Fig. 14). %R.S.D. were below 0.15% in all cases and it was found to be well within accepted criteria < 5%. Values of added and found concentrations were arranged for 1 g of mixture.

Table 13

Results gained for the benzocaine precision

No	Vial	Added c (μg/ml)	Found c (μg/ml)	S.D.	R.S.D. (%)
1	1.1.	119,83	120,28	0,0685	0,0570
2	1.2.	119,83	123,38	0,0804	0,0652
3	2.1.	119,10	116,52	0,1395	0,1197
4	2.2.	119,10	118,82	0,1026	0,0864
5	3.1.	119,08	118,00	0,1003	0,0850
6	3.2.	119,08	119,87	0,1733	0,1446

ACCURACY

Determinations of six samples of 100% concentration in triplicate injection have established set of results for accuracy of benzocaine (Table 14). These results indicate that the method gives recovery from 97.84 – 102.96% (mean value is 100.11%) and %R.S.D. below 0.13%. In general recovery 100 ± 5% and R.S.D. < 5% are

acceptable. Values of added and found concentrations were arranged for 1 g of mixture and calculated according to equation of calibration curve generated from mean values (Fig. 14).

Table 14

Accuracy study of benzocaine

No	Vial	Added c (µg/ml)	Found c (µg/ml)	Recovery (%)	R.S.D. (%)
1	1.1.	119,83	120,28	100,37	0,0476
2	1.2.	119,83	123,38	102,96	0,0544
3	2.1.	119,10	116,52	97,84	0,1005
4	2.2.	119,10	118,82	99,77	0,0725
5	3.1.	119,08	118,00	99,09	0,0714
6	3.2.	119,08	119,87	100,66	0,1214

4.2.2 *p-N*itrobenzoic acid ethylester

SPECIFICITY

Method specificity was verified by comparison of chromatograms of placebo Herplex excipient, Herplex excipient mixed with EPNB and Herplex excipient mixed with benzocaine and EPNB performed with method A.

On
Fig. 15 and
Fig. 16 are shown chromatograms with and without an unknown product with retention time in range of 15.320 – 17.043 min.

In chapter 4.2.1 we have proved that peak is coming from filter paper 388 used for filtration of glacial acetic acid solution.

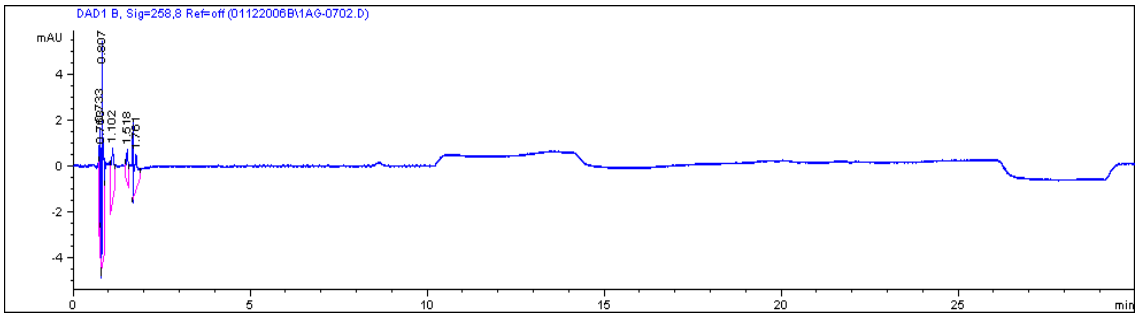


Fig. 15 Chromatogram of Herplex excipient obtained by method A using Cellulose acetate filter for filtration of acetic acid solution

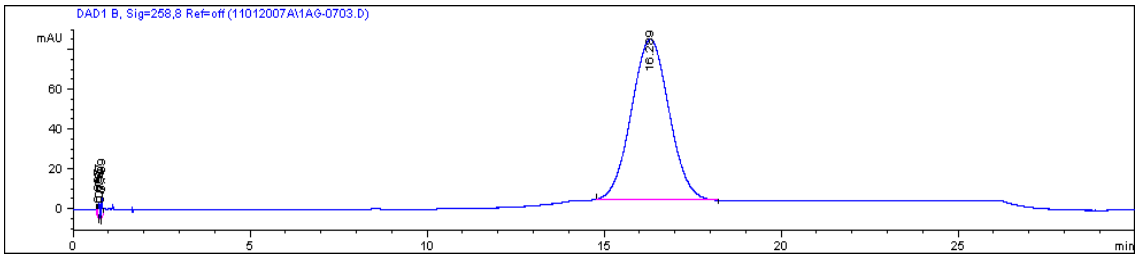


Fig. 16 Chromatogram of Herplex excipient with product from filter paper 388 obtained by method A

p-Nitrobenzoic acid ethylester elutes at 19.428 min. (

Fig. 17) and no interfering peaks in the vicinity of its peak were observed in sample chromatograms (

Fig. 18).

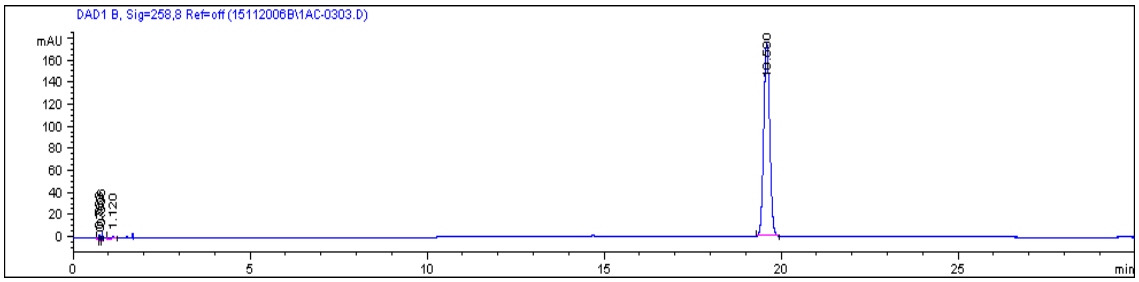


Fig. 17 Chromatogram of Herplex excipient mixed with EPNB obtained by method A

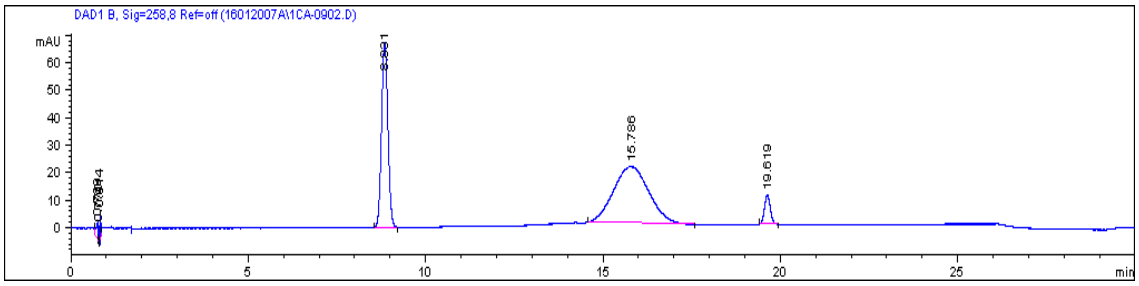


Fig. 18 Chromatogram of Herplex excipient mixed with benzocaine and EPNB in concentration close to LOQ value under the conditions of method A (retention time: benzocaine 8.601, filter paper 388 product 15.786 and EPNB 19.519 min)

LINEARITY

Linear relationship was provided between the peak area of EPNB and the corresponding concentrations, as shown calibration curve (

Fig. 19) of mean peak areas values from obtained results (Table 15). Values of peak areas were adjusted to concentrations stated in table.

Table 15

Values of peak areas and corresponding concentrations for EPNB linearity; solution No 1 (EPNB weigh 20.04 mg) measured two times (sample No 1.1, 1.2), solution No 2 (EPNB weigh 20.00 mg) measured two times (sample No 2.1, 2.2).¨

Concentration	Sample No/Peak area (mAU*s)				Mean
(µg/ml)	1.1.	1.2.	2.1.	2.2.	(mAU*s)
0,50	0,00	0,00	0,00	0,00	0,00
1,00	18,45	18,30	17,97	17,83	18,14
2,00	37,09	36,69	36,67	36,44	36,72
4,00	74,16	74,00	72,69	72,76	73,40
8,00	149,09	148,47	145,38	145,72	147,16
16,00	298,13	296,30	291,07	290,40	293,98
40,00	744,77	740,61	734,11	727,47	736,74
80,00	1491,27	1485,20	1479,50	1465,71	1480,42
160,00	3022,16	2970,55	2981,46	2943,37	2979,38
200,00	3763,60	3727,40	3734,44	3722,68	3737,03

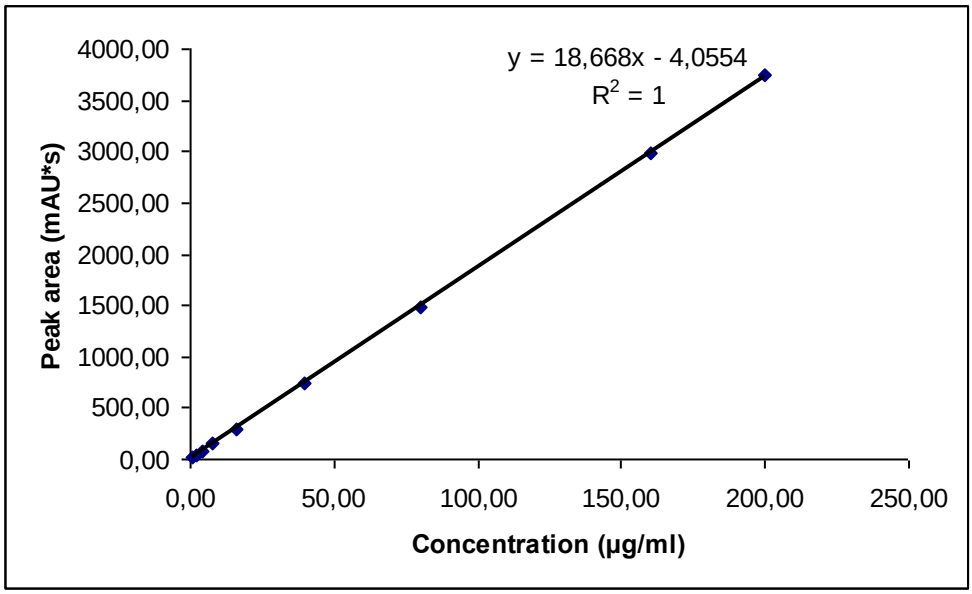


Fig. 19 Calibration curve of EPNB gained from mean values of peak area and corresponding concentrations

The linear regression equation y = 18.668x – 4.40554 and the correlation coefficient R^2 1.0000 were also calculated from mean values. In Table 16 regression equations, y-intercepts and slopes of the regression line of each set of standard samples are reported. The correlation coefficients of each individual measuring as well as correlation coefficient of mean values correspond to the recommended value $R^2 \geq 0.999$.

Table 16

Equation of each set of linearity standard samples of EPNB in the range of 1.0 – 200.0 µg/ml

Sample	Equation for calibration curve	R^2
1.1.	y = 18.846x - 3.5233	1,0000
1.2.	y = 18.614x - 1.5752	1,0000
2.1.	y = 18.072x - 5.2853	1,0000
2.2.	y = 18.542x - 5.8376	0,9999

Equation of calibration curve (
Fig. 20) in a range of concentration from 8 to 200 µg/ml of EPNB was applied for calculation of precision and accuracy studies.

Table 17

Values of peak areas and corresponding concentrations used for calculation of EPNB precision and accuracy studies

Concentration (µg/ml)	Sample No/Peak area (mAU*s)				Mean (mAU*s)
	1.1.	1.2.	2.1.	2.2.	
8,00	143,19	143,34	143,50	143,78	143,45
16,00	287,10	288,75	287,28	288,09	287,80
40,00	719,76	720,49	692,07	720,94	713,31
80,00	1 455,22	1 455,50	1 465,94	1 449,16	1 456,45
160,00	2 931,78	2 915,02	2 990,19	2 915,01	2 938,00
200,00	3 724,52	3 692,08	3 809,28	3 691,97	3 729,46

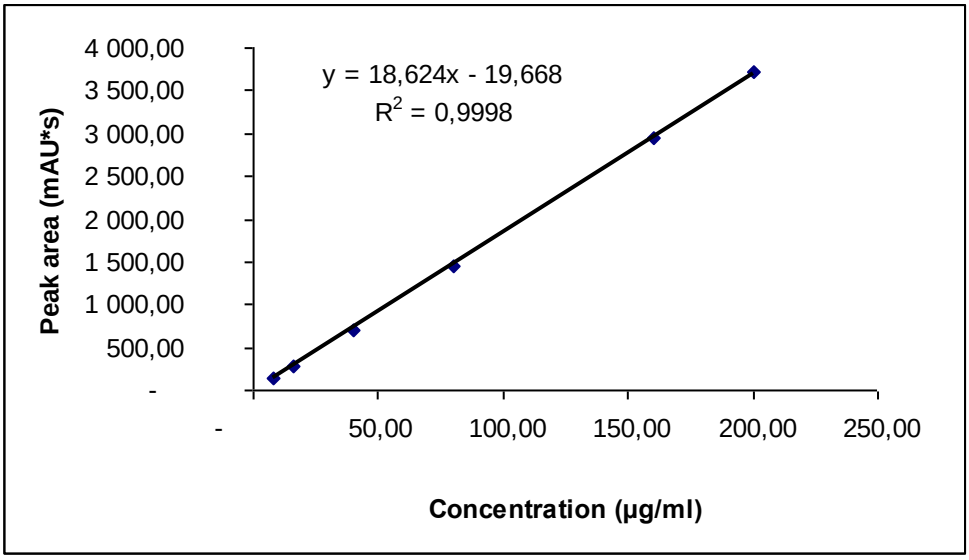


Fig. 20 Calibration curve of EPNB generated from the mean values of peak areas (Table 17) and corresponding concentrations used for precision and accuracy studies

RANGE

The method was found to be linear in the range 1.0 – 200.0 µg/ml (
Fig. 19). These concentrations cover area from LOQ value of EPNB to the concentration used for precision and accuracy tests of EPNB. Concentration 0.5 µg/ml is below the LOQ value of EPNB and this peak area could not be determined.

PRECISION

Method precision or repeatability was studied at 100% concentration of EPNB with six samples and triplicate injection of each sample. The results obtained are presented in Table 18 and were calculated according to equation of calibration curve generated from mean values (

Fig. 20). %R.S.D. were below 0.33% in all cases and it was found to be well within accepted criteria < 5%. Values of added and found concentrations were arranged for 1 g of mixture.

Table 18

Precision results of EPNB calculated according to the equation in

Fig. 20

No	Vial	Added c (µg/ml)	Found c (µg/ml)	S.D.	R.S.D. (%)
1	1.1.	119,54	121,51	0,3902	0,3211
2	1.2.	119,54	124,09	0,2096	0,1689
3	2.1.	117,35	115,89	0,1612	0,1391
4	2.2.	117,35	121,21	0,1810	0,1493
5	3.1.	118,49	114,32	0,3656	0,3198
6	3.2.	118,49	118,80	0,1643	0,1383

ACCURACY

Determinations of six samples of 100% concentration in triplicate injection have established set of results for accuracy of EPNB (Table 19). These results indicate that the method gives recovery from 96.49 – 103.91% (mean value is 100.71%) and %R.S.D. below 0.27%. In general recovery $100 \pm 5\%$ and R.S.D. $< 5\%$ are acceptable. Values of added and found concentrations were arranged for 1 g of mixture and calculated according to equation of calibration curve

Fig. 20.

Table 19

Accuracy study of EPNB calculated according to the equation in

Fig. 20

No	Vial	Added c (µg/ml)	Found c (µg/ml)	Recovery (%)	R.S.D. (%)
1	1.1.	119,54	121,51	101,65	0,2687
2	1.2.	119,54	124,09	103,81	0,1413
3	2.1.	117,35	115,89	98,76	0,2663
4	2.2.	117,35	121,21	103,29	0,1144
5	3.1.	118,49	114,32	96,49	0,2699
6	3.2.	118,49	118,80	100,26	0,1167

LOD and LOQ

Limit of detection and limit of quantification were evaluated according to formula (6 and 7) by means of the standard deviation of the y-intercepts (Table 16) and the slope of calibration curve of EPNB (

Fig. 19). The LOD and LOQ values found for EPNB were 0.3404 µg/ml 1.0315 µg/ml respectively.

4.2.3 *p*-Aminobenzoic acid

SPECIFICITY

Method specificity was verified by comparison of chromatograms of placebo Herplex excipient (

Fig. 21) and Herplex excipient mixed with PABA (

Fig. 22) and chromatogram of Herplex gel (

Fig. 24) performed by method B.

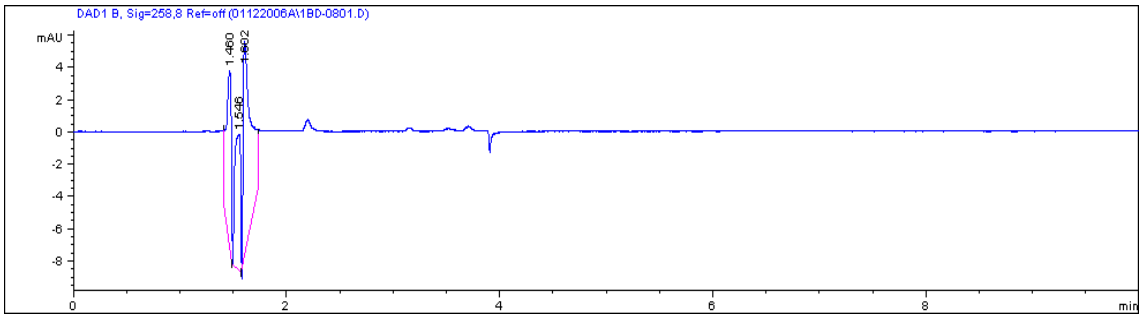


Fig. 21 Chromatogram of placebo Herplex excipient obtained by method B

p-Aminobenzoic acid elutes at 2.192 min (

Fig. 22) and no interfering peaks in the vicinity of this peak were observed on chromatogram of Herplex excipient mixed with PABA (

Fig. 22) or chromatogram of Herplex excipient mixed with PABA, benzocaine and EPNB (

Fig. 23).

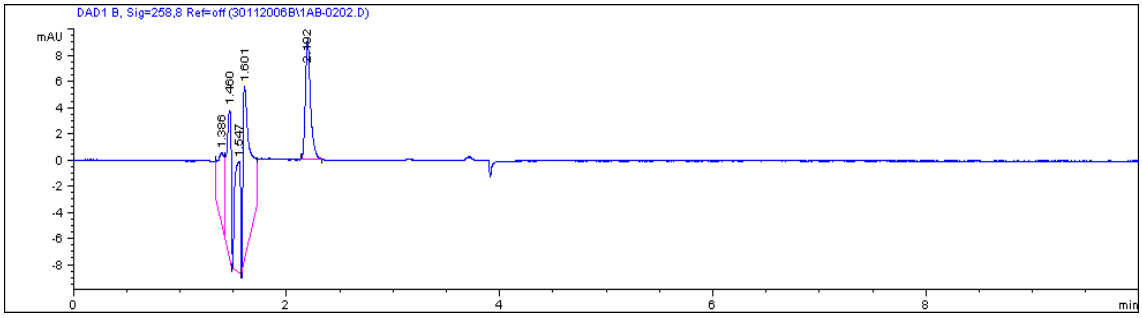


Fig. 22 Chromatogram of Herplex excipient mixed with PABA (concentration close to LOQ value), method B

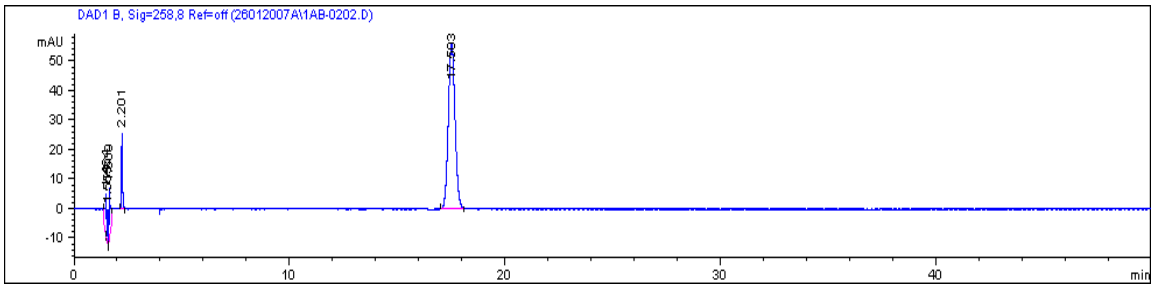


Fig. 23 Chromatogram of Herplex excipient mixed with PABA, benzocaine and EPNB gained by method B prolonged to 50 min (PABA, benzocaine elute at 2.201 and 17.563 min, respectively)

Chromatogram of Herplex gel (

Fig. 24) demonstrates peak of benzocaine (retention time 6.412 min), which comes from previous injection of sample. Peak is recognized after next injection of Herplex gel sample under the conditions of method B. If determination time of method B is

prolonged to 50 min peak of benzocaine is identified after first injection in retention time 17.503 min (

Fig. 25).

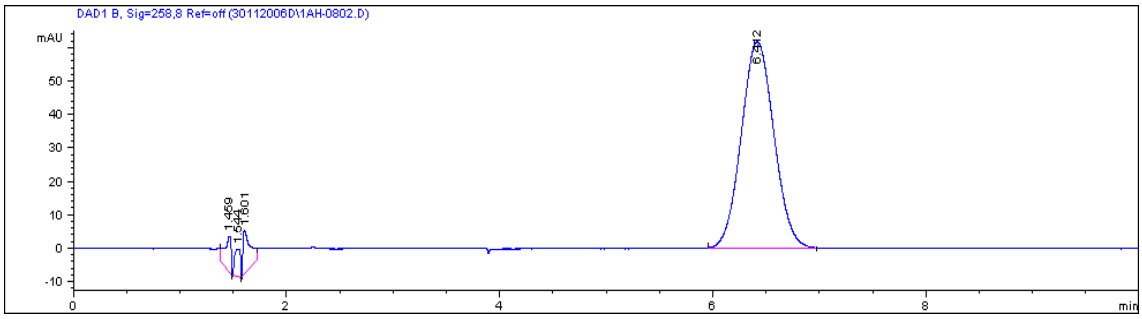


Fig. 24 Chromatogram of Herplex gel with benzocaine peak from previous injection obtained by method B

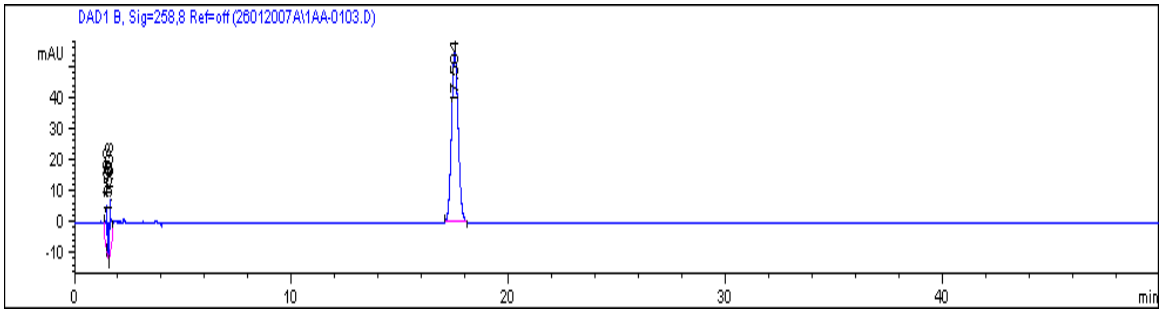


Fig. 25 Chromatogram of Herplex gel gained under the conditions of method B prolonged to 50 min.

LINEARITY

Linear relationship was provided between the peak area of PABA and the corresponding concentrations, as shown calibration curve (

Fig. 26) of mean peak areas values from obtained results (Table 20). Values of peak areas were adjusted to concentrations stated in table.

Table 20

Values of peak areas and corresponding concentrations for PABA linearity; solution No 1 (PABA weigh 20.10 mg) measured two times (sample No 1.1, 1.2), solution No 2 (PABA weigh 20.00 mg) measured two times (sample No 2.1, 2.2).

Concentration (µg/ml)	Sample No/Peak area (mAU*s)				Mean (mAU*s)
	1.1.	1.2.	2.1.	2.2.	
0,50	9,35	9,35	9,92	9,34	9,49
1,00	19,13	19,01	19,08	18,77	19,00
2,00	37,96	37,93	38,12	37,85	37,97
4,00	74,97	75,65	75,34	76,15	75,53
8,00	151,71	151,09	150,84	150,57	151,06
16,00	300,70	301,27	301,75	301,08	301,20
40,00	759,55	753,82	760,65	772,58	761,65
80,00	1520,51	1520,64	1525,42	1569,29	1533,96
160,00	3045,25	3064,40	3133,30	3188,46	3107,85
200,00	3974,09	4000,86	3977,77	4033,69	3996,60

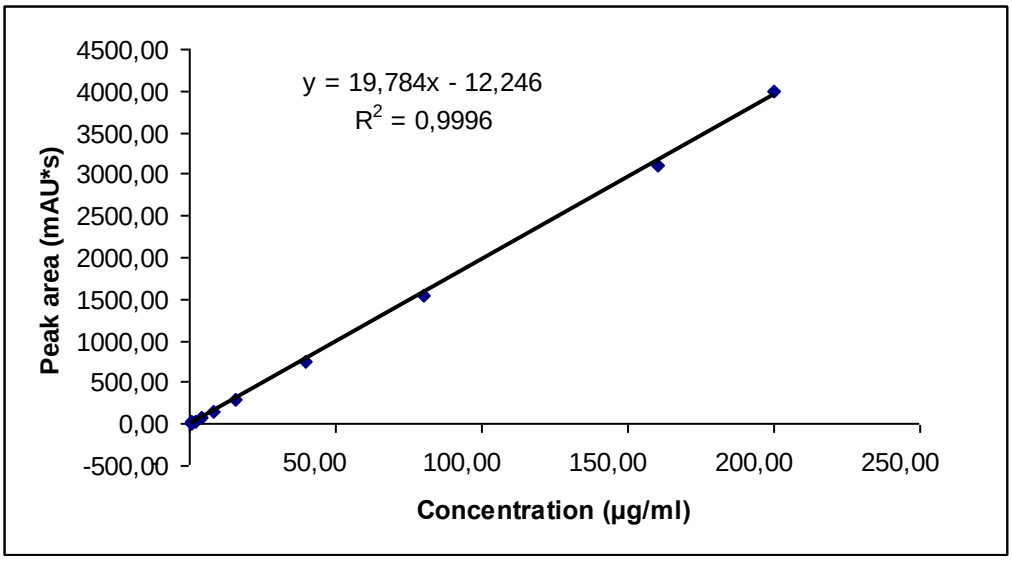


Fig. 26 Calibration curve of PABA generated from the mean values of peak areas and corresponding concentrations

The linear regression equation $y = 19.784x - 12.246$ and the correlation coefficient R^2 0.9996 were also calculated from mean value. The correlation coefficient of mean values as well as correlation coefficients of each individual set of standard samples (Table 21) correspond to the recommended value $R^2 \geq 0.999$. In Table 21 regression equations of each sample are reported.

Table 21

Equation of each set of linearity standard samples of PABA in a range of 0.5 – 200.0 µg/ml

Sample	Equation for calibration curve	R^2
1.1.	y = 19.559x - 11.096	0,9993
1.2.	y = 19.687x - 13.566	0,9993
2.1.	y = 19.782x - 12.136	0,9998
2.2.	y = 20.107x - 12.187	0,9999

Equation of calibration curve (Fig. 27) in a range of concentration from 8 to 200 µg/ml of PABA was applied for calculation of precision and accuracy studies.

Table 22

Values of peak areas and corresponding concentrations used for calculation of PABA precision and accuracy studies

Concentration (µg/ml)	Sample No/Peak area (mAU*s)				Mean (mAU*s)
	1.1.	1.2.	2.1.	2.2.	
8,00	136,14	136,16	140,56	141,44	138,57
16,00	273,76	273,58	282,44	284,27	278,51
40,00	686,95	685,96	710,78	712,26	698,99
80,00	1 377,01	1 377,44	1419,15	1434,12	1 401,93
160,00	2 754,88	2 768,25	2862,72	2908,06	2 823,48
200,00	3 584,99	3 547,08	3643,14	3644,80	3 605,00

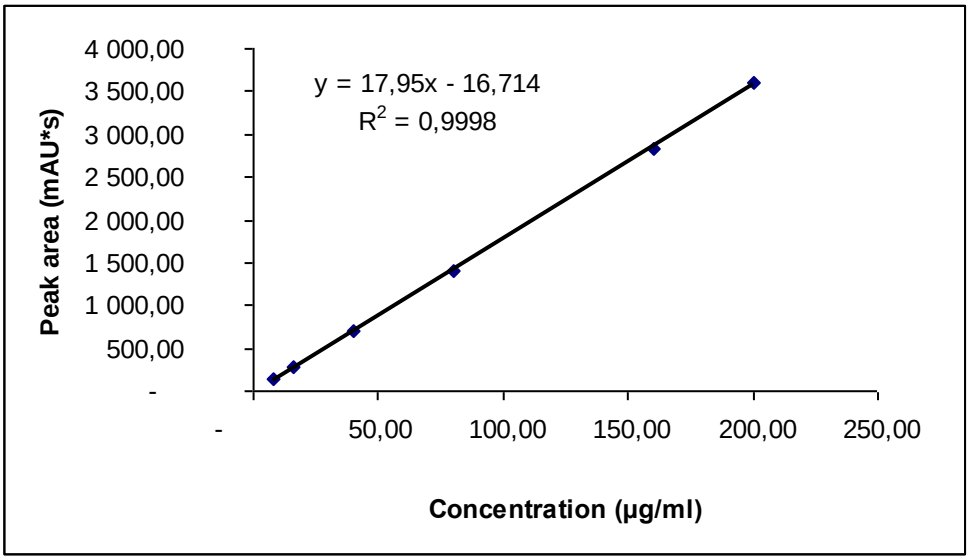


Fig. 27 Calibration curve of PABA generated from the mean values of peak areas (Table 22) and corresponding concentrations used for precision and accuracy studies.

RANGE

The method was found to be linear in the range of 0.5 – 200.0 µg/ml (
Fig. 26). These concentrations cover area from the LOQ value of PABA to the concentration used for precision and accuracy tests of PABA.

PRECISION

Method precision or repeatability was studied at 100% concentration of PABA with six samples and triplicate injection of each sample. The results obtained are presented in Table 23 and were calculated according to equation of calibration curve generated from mean values. %R.S.D. were below 0.11% in all cases and it was found to be well within accepted criteria < 5%. Values of added and found concentrations were arranged for 1 g of mixture.

Table 23

Precision results of PABA calculated according to the equation in Fig. 27

No	Vial	Added c (µg/ml)	Found c (µg/ml)	S.D.	R.S.D. (%)
1	1.1.	79,83	80,03	0,0650	0,0812
2	1.2.	79,83	81,65	0,0330	0,0404
3	2.1.	80,11	81,19	0,0791	0,0974
4	2.2.	80,11	81,78	0,0327	0,0400
5	3.1.	80,01	80,59	0,0293	0,0364
6	3.2.	80,01	81,73	0,0825	0,1010

ACCURACY

Determinations of six samples of 100% concentration in triplicate injection have established set of results for accuracy of PABA (Table 24). These results indicate that the method gives recovery from 100.21 – 102.24% (mean value is 101.47% and %R.S.D. below 0.13%. In general recovery $100 \pm 5\%$ and R.S.D. $< 5\%$ are acceptable. Values of added and found concentrations were arranged for 1 g of mixture and calculated according to equation of calibration curve on Fig. 27.

Table 24

Accuracy study of PABA calculated according to the equation in Fig. 27

No	Vial	Added c (µg/ml)	Found c (µg/ml)	Recovery (%)	R.S.D. (%)
1	1.1.	79,83	80,03	100,25	0,1017
2	1.2.	79,83	81,65	102,28	0,0506
3	2.1.	80,11	81,19	101,34	0,1216
4	2.2.	80,11	81,78	102,09	0,0499
5	3.1.	80,01	80,59	100,73	0,0455
6	3.2.	80,01	81,73	102,15	0,1262

LIMIT OF DETECTION

Limit of detection and limit of quantification were evaluated according to formula (6 and 7) by means of the standard deviation of the y-intercepts (Table 21) and the slope of calibration curve of PABA (

Fig. 26). The LOD and LOQ values found for PABA were 0.1691 µg/ml and 0.5123 µg/ml, respectively.

4.3 EXTRACTION FROM HERPLEX GEL

The method was examined in the assays of the 11 batches (batches A – K) of Herplex gel exposed in 3.1.3. In Table 25 dates of measurement and expiration dates of individual batches are presented.

Table 25

List of batches used for determination

Sample	Batch	Expiration	Date of measurement
A	No 07670904	09/2005	12/2006
B	No 02870306	03/2007	11/2006
C	No 09341105	11/2006	11/2006
D	No 06010706	07/2007	12/2006
E	No 09771206	12/2007	01/2007
G	No 09781206	12/2007	01/2007
I	No 05990706	07/2007	01/2007
J	No 06000706	07/2007	01/2007
K	No 06010706	07/2007	01/2007

Assay samples for determination were consisted of two samples of each batch (sample A1, A2; B1, B2; … till K1, K2). Each of two samples had parallel vials (sample A1 had vials No 1 and 2 and so on). Accurately weighed amounts of the individual gels as well as the approaches for extraction of gel (method A and method B) are described in chapter 3.4.4. All batches were analysed under the conditions of method A and method B.

Table 26

% recovery of benzocaine (BZC) from Herplex gels obtained using method A (batches A-K as listed in

Table 7)

Sample	Theoretical c of BZC (µg/ml)	Vial No	Mean of peak areas (mAU*s)	Measured c of BZC (µg/ml)	% recovery
A1	141,30	1	1 082,05	135,47	95,87
		2	1 142,03	142,92	101,15
A2	139,74	1	1 104,52	138,26	98,94
		2	1 026,36	128,55	91,99
B1	122,24	1	968,58	121,37	99,29
		2	951,50	119,25	97,55
B2	131,03	1	1 067,88	133,71	102,05
		2	1 078,52	135,03	103,06
C1	120,22	1	961,79	120,53	100,26
		2	949,86	119,05	99,03
C2	126,19	1	1 023,88	128,24	101,63
		2	1 023,16	128,15	101,55
D1	122,20	1	916,42	114,89	94,02
		2	935,91	117,32	96,01
D2	120,42	1	962,05	120,56	100,12
		2	973,45	121,98	101,29

E1	119,04	1	943,63	118,27	99,36
		2	944,75	118,41	99,47
E2	127,61	1	1 023,23	128,16	100,43
		2	1 037,91	129,99	101,86
F1	120,85	1	956,69	119,90	99,21
		2	988,52	123,85	102,48
F2	124,98	1	1 003,65	125,73	100,60
		2	1 029,43	128,93	103,16
G1	118,68	1	940,14	117,84	99,29
		2	951,37	119,24	100,47
G2	111,72	1	889,77	111,58	99,88
		2	913,02	114,47	102,46
H1	145,31	1	1 133,50	141,86	97,63
		2	1 166,23	145,93	100,43
H2	136,90	1	1 108,00	138,69	101,31
		2	1 122,61	140,51	102,64
I1	116,66	1	928,93	116,45	99,81
		2	941,84	118,05	101,19
I2	122,47	1	999,81	125,25	102,27
		2	1 007,48	126,21	103,05
J1	118,99	1	917,67	115,05	96,69
		2	934,43	117,13	98,44
J2	124,52	1	987,98	123,78	99,41
		2	998,36	125,07	100,44
K1	133,96	1	983,56	123,23	92,00
		2	1 062,39	133,03	99,31
K2	134,48	1	1 030,71	129,09	95,99
		2	1 073,29	134,38	99,92

Table of reports (Table 26) indicates amount of recovery of benzocaine from Herplex gels (method A). All examined batches contain benzocaine in a range of 91.99 - 103.16% of label value. The mean value of recovery was 99.61% (calculated from results of all batches).

USP 22 recommends content of not less than 90.0% and not more than 110.0% of the labelled amount of benzocaine in benzocaine ointments and creams.

None of the samples (batches) has contained evaluated degradation products EPNB or PABA.

4.4 THERMAL STABILITY OF HERPLEX GEL

Thermal stability study of Herplex gel was performed by comparison of results obtained from Herplex gel batch No 09771206, sample 1 and 3 (E1 and F1 respectively) stored at different temperature for 7 days.

Amount of Herplex gel used for stability study is stated in chapter 3.4.4 as well as extraction procedures for benzocaine and EPNB (method A) and PABA (method B). Sample E1 was stored at room temperature for 7 days and sample F1 was stored at temperature 100 ˚C for 7 days.

Table 27 shows the results obtained by using method A for extraction of benzocaine and EPNB. After 7 days at temperature 100 ˚C degradation product EPNB was not found in the sample. However by increasing temperature at 100 ˚C was evoked decrease of benzocaine amount in a gel to approximately 50% of intended amount.

Table 27

% benzocaine recovery results of thermal stability study of Herplex gel (batch No 09771206) after 7 days, established by method A (sample E1 stored at room temperature and sample F1 stored at temperature 100 ˚C)

Sample	Theoretical c of BZC (µg/ml)	Vial No	Mean of peak areas (mAU*s)	Measured c of BZC (µg/ml)	% recovery
E1	136,76	1	1 033,35	129,42	94,63
		2	1 028,46	128,81	94,19
F1	110,12	1	476,73	60,27	54,73
		2	498,18	62,94	57,15

By performance of PABA extraction (method B) was established that after 7 days at temperature 100 ˚C degradation product PABA has appeared in a Herplex gel in concentration approximately 0.92 µg/ml (Table 28) calculated according to equation in Fig. 27.

Table 28

Results of thermal stability study of Herplex gel (batch No 09771206) after 7 days, established by method B (sample E1 stored at temperature 25 ˚C and sample F1 stored at temperature 100 ˚C)

Sample	Vial No	Mean of peak areas (mAU*s)	Measured c of PABA (µg/ml)
E1	1	-	-
	2	-	-
F1	1	5,89	0,92
	2	5,71	0,91

4.5 DEGRADATION OF BENZOCAINE

Calculation of benzocaine amount, which has to be degraded for determination of its degradation products EPNB and PABA was establish according to formula (14).

$$n = \frac{m}{M} \qquad (14)$$

n chemical amount (mol)

m total mass (g)

M molar mass (g/mol)

M_{BZC} = 165.192 g/mol

M_{EPNB} = 195.175 g/mol

M_{PABA} = 137.138 g/mol

It was calculated that 1 mol of benzocaine degrades to 1 mol of its degradation product (EPNB or PABA). For determination of 1.0315 µg/ml EPNB (LOQ value of EPNB obtained before) 0.8730 µg/ml benzocaine has to be degraded. It responds $3.6375*10^{-3}$ µg benzocaine contained in 1 g of Herplex gel. And for determination of 0.5123 µg/ml PABA (LOQ value of PABA obtained before) 0.6171 µg/ml benzocaine has to be degraded. It responds $3.8569*10^{-4}$ µg benzocaine contained in 1 g of Herplex gel.

5 CONCLUSION

It was established the new HPLC method for determination and quantification of benzocaine and its two degradation products, *p*-nitrobenzoic acid ethylester and *p*-aminobenzoic acid, in Herplex gel.

The new HPLC method is consisted of two procedures (A and B) for extraction and determination of individual substances of gel since conditions and extraction procedure of benzocaine and EPNB is not sufficiently effective for quantification of PABA.

The determination was performed with HPLC system Agilent 1100 Series, Agilent Technologies and with the column Eclipse XDB – C 18 (150 x 4.6 mm, 5 μm). The mobile phase consisted of a mixture of methanol and glacial acetic acid solution (10%, v/v) at different proportion according to a time-schedule programme (procedure A for benzocaine and EPNB) and at proportion 10% of methanol: 90% of glacial acetic acid solution (procedure B for PABA), pumped at a flow rate of 2.0 ml/min and 1.0 ml/min respectively. The DAD detector was set at 258 nm. Determination time was 30 min and 10 min respectively. Injection volume was 10 μm and thermostat was equilibrated at 40 ºC.

The HPLC method has been evaluated over the linearity, precision, accuracy and specificity. The UV spectra detection was measured too. The linearity was determined with the range of benzocaine concentration 8 - 160 μg/ml and 0.5 – 200.0 μg/ml of EPNB and PABA concentration and with the correlation coefficient higher than 0.9990 for individual substances. Results of precision and accuracy tests (%recovery and R.S.D.) of all substances were bellow the acceptable value 5%. The LOD value was 0.3404 μg/ml and the LOQ value was 1.0315 μg/ml for EPNB and for PABA 0.1691 μg/ml and 0.5123 μg/ml respectively.

For extraction from gel 9 batches of Herplex gel with different ages were used. All examined batches contained benzocaine in a range of 91.99 – 103.16% of label value.

In thermal stability study was found that content of benzocaine in Herplex gel fell after 7 days at temperature 100 ºC to 50% of label value and PABA has appeared in Herplex gel in concentration approximately 0.92 μg/m.

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